Frontiers in Science and Engineering
International Journal

Edited by The Hassan II Academy of Science and Technology of Morocco

Life Sciences (Medicine, Health, Agriculture, Biology, Genetics)

Contents

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All papers are submitted to the normal peer-review process. Publication criteria are based on :
i) Novelty of the problem or methodology and problem solving, **ii**) Salience of the approach and solution technique, **iii**) Technical correctness and outputs, **iv**) Clarity and organization.

Papers are first reviewed by the Executive Board Director who receives the paper and, if relevant in terms of the overall requirements, it is then proposed to one of the most appropriate associate editor on the field who will select 2 to 3 expert reviewers. Electronic printing will allow considerable time savings for submission delays which will be reduced drastically to less than three to six months. Prospective authors are therefore invited to submit their contribution for assessment while subjected to similar quality criteria review used in paper journals.

Authors are notified of acceptance, need for revision or rejection of the paper. It may be noted that papers once rejected cannot be resubmitted. All the details concerning the submission process are described in another section. This electronic journal is intended to provide :

- the announcement of significant new results,
- the state of the art or review articles for the development of science and technology,
- the publication of proceedings of the Academy or scientific events sponsored by the Academy,
- the publication of special thematic issues.

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The journal covers the established disciplines, interdisciplinary and emerging ones. Articles should be a contribution to fundamental and applied aspects, or original notes indicating a significant discovery or a significant result.

The topics of this multidisciplinary journal covers amongst others :
Materials Science, Mathematics, Physics, Chemistry, Computer sciences, Energy, Earth Science, Biology, Biotechnology, Life Sciences, Medical Science, Agriculture, Geosciences, Environment, Water, Engineering and Complex Systems, Science education, Strategic and economic studies, and all related modeling, simulation and optimization issues, etc. ...

Once, a certain number of papers in a specific thematic, is reached, the Academy might edit a special paper issue in parallel to the electronic version.

FOREWORD

Cancer is a major public-health problem in Africa. However the advances made in the treatment of these range of diseases over the past decade are hopeful and the emergence of targeted therapies has changed the evolution of some cancers, known to have a poor prognosis. These therapies have a very high cost which makes them out of reach of the majority of patients in developing countries. There are typically three main types of cancer treatment through surgery, radiotherapy and chemotherapy. The latter is undoubtedly saving lives but at the cost of significant toxicity. The risk of toxicity associated with chemotherapy must therefore be weighed against the risk of cancer progression due to the discontinuation of systemic therapy.

Thanks to precision medicine, a revolution is also in progress. Targeted therapies are thus currently carried out and help understanding the mechanisms of cancer-cell function. Targeted therapy uses a "selective" drug that attacks cancer cells by spotting a specific target. This target can be a receptor, a gene or a protein, and the targeted action must intervene at a precise stage of the development of the tumor cell, protecting as much as possible the healthy cells.

Targeted therapy is mainly involved in signal transduction pathways, which controls cell multiplication. The so-called tyrosine kinase pathway is the best known up to date. Monoclonal antibodies or enzymatic inhibitors can block this pathway. By acting on specific receptors, these drugs can block the growth of cancer cells, by preventing the tumor from inducing its own vascularization; or they can stimulate the immune system of the patient against cancer cells. Also should be mentioned the recently approved immunotherapy treatment of non small-cell metastatic bronchial-cell cancers. Targeted therapy can also control cancer-cell death, enhancing apoptosis or the natural death of the cell. Some products are being developed for the treatment of cancers of the upper aero-digestive tract (head and neck). These are examples of targeted therapies, being tried and used up to date and which have significantly improved the treatment of cancers. This was the focus of the three-day Summer School (16-18 July 2018), organized by the Hassan II Academy of Science and Technology (Life Sciences and Biotechnology section), in close cooperation with the Al Akhawayn University at Ifrane, Morocco.

The program also aimed to the discuss the situation in Morocco and Africa, as well as on how to make the new therapies available to the patients. The participants in the summer school included, in addition to renowned experts from Morocco, Africa and other countries, 15 young researchers (including two from Africa) and 25 senior researchers from various Moroccan institutions.

This special issue of Frontiers in Science and Engineering includes eight articles or presentations of from this summer school.

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Members of the Life Sciences and Biotechnology Section

COMBATING CANCER: NEW APPROACHES THROUGH IMPROVED DIAGNOSIS AND IMMUNOTHERAPY

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Background

Hopes were high when the president of the United States of America, Richard Nixon, declared war on cancer in 1971. But over 47 years later more than 15 million people worldwide still die from the diseases that fall under the broad name of cancer. In the case of the United States, for instance, the estimated number of new cancer diagnoses in 2018 was evaluated at 1,735,350. The number of deaths per day from cancer was estimated at 1,700; lung, prostate, breast and colorectal cancers accounted for about 45%; the estimated number of new breast cancer cases, the most common type, was 265,120 in 2018; the mortality rate in the United States is currently 164 in 100,000 people a year.

It is nevertheless true that cancer has become less deadly for Americans over the past 25 years (1990-2014) – a decline of 25%, largely due to a declining number of smokers, earlier diagnosis and some significant improvements in treatment. But the problems – and the costs – are still overwhelming: *ca*. US$147.3 billion have been spent on cancer care in 2018, and almost US$50 billion of that amount was spent on drugs. It has been calculated that the average amount an insurer agreed to pay for a year of treating an advanced breast cancer patient, was US$134,882, according to a 2016 study. The average allowed coverage for early-stage disease was US$60,637. Some treatments could reach much higher amounts: e.g. US$475,000 in 2018 for one of the most expensive cancer drugs, Kymriad, produced by the big pharmaceutical company Novartis; this drug uses the immune system of the patient to destroy cancer cells in the bloodstream and it was considered a breakthrough to combat leukemia (Cohen, 2018).

* February 2019.

The Hassan II Academy of Science and Technology, in close partnership with Al Akhawayne University (Ifrane, Morocco), have organized a "summer school" on Advances in cancer therapies, at the University campus, from 16 to 18 July 2018. The school was attended by about 20 PhD students working on the subject and are already advanced in their research work, as well as by a group of experts from Morocco and abroad. The proceedings of the school are to be published by the Academy and the University, while a summary of the main outcomes of the event will be published in the Bulletin of the Academy (a semestrial publication).

This review article has been inspired by my attendance of, and participation in, the summer school.

Can it therefore be said that the war on cancer has failed? No, says Paul Marks in his book *On the Cancer Frontier: One Man, One Disease*, *and a Medical Revolution*, co-authored with James Sterngold, and published in 2014. According to the former head of Memorial Sloan-Kettering Cancer Center in New York, the goal should be containment not victory, because the enemy is uniquely intractable. "Medical science has never faced a more inscrutable, more mutable, or more ruthless adversary," wrote P. Marks who has taken part in many of the developments that have enhanced the understanding of the disease (*The Economist*, 2014; Sasson 2016).

Researchers now understand that cancer is not one disease, but essentially hundreds. Thus the very notion of a single cure – or as President Barack Obama put it, making "America the country that cures cancer once and for all" – seems misleading and outdated. "Cancer is way more complex than anyone had imagined in 1970" (at the time of President R. Nixon), stated José Baselga, president of the American Association for Cancer Research and chief medical officer at Memorial Sloan-Kettering Cancer Center in New York (Kolata and Harris, 2016).

Cancer is actually a term that embraces hundreds of specific ailments caused by an even larger number of genetic and epigenetic traits. Yet not all cancers are caused by just one agent – for instance a virus or a bacterium that can be destroyed. Cancer is an intricate and potentially lethal collaboration of genes that do not function correctly, of cell-growth inhibitors gone missing, of hormones and epigenomes changing and cells escaping any control. "This disease is much more complex than we have been treating it, and the complexity is stunning," stated Phillip Sharp of the Massachusetts Institute of Technology (MIT), a Nobel Laureate in Medicine or Physiology (in 1993 for the discovery of introns), who studies the genetics of cancer (Saporito, 2013).

Only *ca*. 10% of cancers are hereditary, but all cancers are genetical, i.e. associated with DNA instability often caused by environmental factors (e.g. tobacco and smoking, alcohol, food, sun exposure, chemicals, viruses), and consequently not inherited from our genitors. In other words cancer(s) is (are) always a DNA disease or disorder that is generally acquired (in *ca*. 90% of cases) [Alexandre, 2001].

The probability of an individual developing invasive cancer at some point in life is one out of three. There are in addition disparities within populations. For instance, in the United States, 14% is the percentage by which cancer deaths among non-Hispanic black people exceeded those among non-Hispanic white people in 2015, on average. In Washington, D.C., and some States such as Louisiana, Kansas, Illinois and California, the cancer mortality rate was 50% higher among black people compared with white people. Also, 60% of black men are suffering from lung cancer, who are diagnosed when the disease is already advanced, compared with 50% of white men with the same cancer. Mortality rates due to cancer among black women were in the United States *ca*. 181 per 100,000, compared with *ca*. 155 per 100,000 among white women. Similarly the annual rate of death from all cancers among Northern Plains American Indians between 1999 and 2009 was estimated at 338 per 100,000, compared with 223 per 100,000 among white people during those years (Cohen, 2018).

In France, since the 1970s the increase in smoking among women has had a major impact on the morbi- and morta-lity associated with the consumption of tobacco, according to the results of a study published in the *Bulletin épidémiologique hebdomadaire* (Weekely Epidemiological Bulletin) of 30 October 2018 devoted to tobacco consumption and smoking. Thus, the impact on lung cancer has been increased by 72% among women between 2002 and 2012. The number of deaths due to smoking doubled among women, between 2000 and 2014: 8,027 deaths in 2014, i.e. 3.3% of total mortality.

When cancer is caught earlier, the treatment is usually easier and outcomes better; henceforth the primary importance of an early and accurate diagnosis. On the other hand, new hopes are arising for treatment, using immunotherapy. Indeed, researchers are developing more targeted drugs and immune therapies, and say in the futures they expect to hit cancers with several such treatments at once, much the way AIDS/HIV was treated when researchers developed drugs to strike the virus at its vulnerable sites.

May be this background or introduction could be concluded by the statement made by José Baselga: "We are in a situation now where we can really make an impact." "But at this point, funding matters," he added (Kolata and Harris, 2016). In fact, during President B. Obama's administration, funding for the National Cancer Institute was significantly increased after years of static budgets. During several meetings held with Vice-President Joe R. Biden, discussions aimed at improving federal investment and support for cancer research and treatment. The researchers offered a number of ideas on how the vice-president of the United States could be helpful, not simply for new research, but for making sure what is being discovered at such a rapid pace today is not squandered, that patients who could benefit were actually helped (Kolata and Harris, 2016). This indeed raises the crucial issue of the accessibility of the patient to new very expensive treatments (see below).

Improvements in diagnostics tools

Designing one blood test to select several cancers

The ultimate objective of oncologists is to design a universal test to detect all cancers, at an early stage. Over the last three or four years (2014-2017), breakthroughs have been achieved with the development of tests based on the analysis of tumoural DNA in the bloodstream. In France, for instance, several teams are working on the subject, such as those of Thierry Freburg, a professor at the department of genomics and personalized medicine, university hospital of Rouen, Normandy, and of Pierre-Laurent Puig, a professor of oncology at the European Hospital Georges-Pompidou in Paris. The latter has developed a blood test for the detection of malignant tumours in lung and pancreas (Benkimoun, 2018).

It should be recalled that fragments of DNA are liberated from dying malignant cells into the bloodstream. Hence the use of the phrase "liquid biopsy" to define the technique to detect them. These circulating DNAs have a small size about 100 to 150 nucleotide pairs. They are amplified before being analyzed; hence their name of *amplicons*. The identification of mutations in the circulating tumoural DNA could help detect a cancer at an early stage, when the tumour cannot be perceived through the use of conventional methods. To that end the technique should have a great sensitivity, because of the very small quantities of circulating tumoural DNA. Also because the technique should enable make the difference between tumoural DNA and genetic material shed out of normal tissues (and thus avoid false positive results) [Benkimoun, 2018].

In 2018 Joshua Cohen of Johns Hopkins University School of Medicine Ludwig Center for Cancer Genetics and Therapeutics, Baltimore, and many of his colleagues, from Baltimore and other research centres in the United States and Australia (Melbourne), described a blood test that can detect eight common cancer types through the assessment of the levels of circulating proteins and mutations in cell-free DNA. The test, called CancerSEEK, was applied to 1,005 patients with non-metastatic, clinically detected cancers of the ovary, liver, stomach, pancreas, oesophagus, colorectum, lung or breast; these cancers were responsible of a total of 360,000 deaths per annum, i.e. 60% of the deaths due to cancer in the United States. CancerSEEK tests

were positive in a median of 70% of the eight cancer types. The sensitivities ranged from 69% to 98% for the detection of five cancer types (ovary, liver, stomach, pancreas and oesophagus) for which they were no screening tests available for average-risk individuals. The specificity of CancerSEEK was greater than 99%: only 7 of 812 healthy controls scored positive. In addition CancerSEEK localized the cancer to a small number of anatomic sites in a median of 83% of the patients (Cohen et al., 2018). Among the senior co-authors of the article published in *Science* (23 February 2018), Cristian Tomasetti (of Johns Hopkins University School of Medicine Sidney Kimmel Cancer Center, Baltimore) and Bert Vogelstein (of Johns Hopkins University School of Medicine Ludwig Center for Cancer Genetics and Therapeutics, Baltimore) had published in 2015 and 2017 their controversial data regarding the occurrence of cancers, resulting much more from haphazard mutations than from environmental or behavioural factors (Tomasetti and Vogelstein, 2015).

The specificity of the CancerSEEK test "would permit a better orientation of the follow-up to the disease, a real focus on the appropriate treatment and the elimination of many useless checks," stated Alexandra Martins of the department of genomics and personalized medicine, at the French National Institute for Health and Medical Research –INSERM– CR1, Rouen, Normandy (Benkimoun, 2018).

Finally, the American and Australian researchers have emphasized the relatively modest cost of the cancer SEEK test, the patent of which was owned by Johns Hopkins University, i.e. US$500 (or €408). This cost is comparable to other existing detection tests for one single cancer (e.g. coloscopy). The authors also underlined that their test was not replacing other means of cancer detection, but it aimed to complement them. According to Thierry Frebourg, "this study confirms that circulating tumour DNA is a cancer-detection means, as well as the use of combining genetic and proteic makers in order to optimize the test. The technique could still be improved thanks to the analysis of metabolites as well as to taking account of epigenetics due to DNA methylation (which has an influence on the expression of genes)". But by all means, according to Pierre Laurent-Puig, research should above all focus on the detection of cancers at an early stage (stage 1, i.e. a unique and small-sized tumour) among asymptomatic individuals (Benkimoun, 2018).

Genetic test that can spare breast cancer chemotherapy

Ca 250,000 women in Europe, North America and Japan are diagnosed each year (e.g. 2018) with the common type of breast cancer and the vast majority of them are treated with chemotherapy after surgery, subjecting them to toxic side-effects such as nausea and hair loss. But a trial of more than 10,000 patients suggested that *ca*. 70% of these women should not be treated with chemotherapy because it does not improve their survival prospects. They can be treated solely with a milder hormone therapy (Crow, 2018).

A genetic test called Oncotype DX, developed by Genomic Health, a Californian diagnostics company, can be used to predict accurately which women would benefit from chemotherapy. The results of a study, funded by the National Cancer Institute (National Institutes of Health) and also supported by the Breast Cancer Research Foundation and the US Postal Service Breast Cancer Stamp, and concerning a phase-III clinical trial, were presented at the American Society of Clinical Oncology (ASCO) Annual Meeting Plenary Session on 3 June 2018 by Sparano et al. (2018). The trial showed that most women with hormone receptor-positive, HER2-negative, axillary node-negative early-stage breast cancer and a mid-range score on a 21-tumour gene expression assay (Oncotype DX Breast Recurrence Score) *do not need chemotherapy* after surgery. The study found no improvement in disease-free survival when chemotherapy was added to hormone

therapy in this group, which accounts for *ca*. two-thirds of women participating in the trial. This is the largest breast-cancer-treatment trial ever conducted, and the first precision-medicine trial ever done, according to Sparano et al. (2018).

Joseph A. Sparano, associate director for clinical research at the Albert Einstein Cancer Center and Montefiore Health System in New York, and vice-chair of the ECOG-ACRIN Cancer Research Group which designed and conducted the study, stated: "Half of all breast cancers are hormone receptor-positive, HER2-negative, and axillary node-negative. Our study shows that chemotherapy may be avoided in about 70% of these women when its use is guided by the test, thus limiting chemotherapy to the 30% who, we can predict, will benefit from it" ... "Before TAILORx (Trial Assigning Individualized Options for Treatment) there was uncertainty about the best treatment for women with a mid-range score of 11-25 on the Oncotype DX Breast Recurrence Score test. The trial was designed to address this question, and provides a very definitive answer. Any woman with early-stage breast cancer 75 years or younger should have the test and discuses the results of TAILORx with her medical doctor to guide her decision regarding chemotherapy after surgery to prevent recurrence."

The TAILORx enrolled 10,273 women with hormone receptor-positive, HER2-negative, axillary node-negative breast cancer – the most common type of breast cancer. Of those, 6,711 had a mid-range recurrence score of 11-25 and were randomly assigned to receive hormone therapy alone or hormone therapy and chemotherapy. Based on evidence from several prior studies, the 21-gene expression assay is widely used to provide prognostic information about the risk of breast cancer recurrence within ten years, and to predict which patients are most likely to derive a large benefit from chemotherapy. The test is performed on a tumour biopsy sample. Women with a low score (0-10) typically receive only hormone therapy and those with a high score (20-100) receive hormone therapy and chemotherapy (Sparano et al., 2018).

According to Sparano et al. (2018), their findings suggest that chemotherapy may be spared in:
- all women older than 50 years with hormone-receptor positive, HER2-negative, node-negative breast cancer, and a recurrence score of 0 to 25 (*ca*. 85% of women with breast cancer in this age group);

- all women 50 years or younger with hormone-receptor positive, HER2-negative, node-negative breast cancer and a recurrence score of 0 to 15 (*ca*. 40% of women with breast cancer in this age group).

Breast cancers and epigenetics

Ca. 5% to 10% of breast cancers are hereditary, according to the French National Cancer Institute (INCa). Part of these family cancers are explained today by genetic mutations, among which the mostly known are those affecting two genes, BRCA1 and BRCA2. Thus 40% to 85% of women with these mutations will develop a breast cancer before the age of 70 years, compared to 10% in the general population. Although not very frequent, these hereditary forms of cancer are affecting numerous women in France, where *ca*. 54,000 new cases of breast cancer are registered every year (Rosier, 2018).

The fact that a mutation in the gene BRCA is found in 10% to 20% of women belonging to these families at risk, has challenged the work of geneticists. According to Olivier Caron, head of the department of oncogenetics at the Gustave-Roussy Institute, Villejuif, south of Paris, "the challenge is to discover other changes in the genome that explain these family records". In particular one tries to understand the role of "epimutations" in the hereditary transmission of these cancers. Epimutations are changes in gene activity that are transmitted without any modification of the DNA sequence. Such epimutations could be transmitted from one generation of individuals

to another. In plants this is quite frequent, while in mammals and humans the well-identified cases of transmission from one generation to another remain exceptional, because these genetic changes are wiped out during the production of gametes or during the fertilization process (Rosier, 2018).

French researchers belonging to the Institut Curie Research Centre, the National Scientific Research Centre (CNRS, French acronym), the National Institute for Health and Medical Research (INSERM), and other cancer-research centres and university laboratories, led by Edith Heard, Anne Vincent-Salomon and Marc-Henri Stern, published in 2015 their results on the epigenetic instability of the inactive X chromosome in breast tumours and cell lines (Chaligné et al., and Heard, Vincent-Salomon, Stern, 2015). The inactive X chromosome (Xi), also known as the Barr body, provides an outstanding example of an epigenetic nuclear landmark that is disrupted by cancer. The disappearance of the Barr body in breast tumours was noted several decades ago, but to date, only genetic instability had been clearly demonstrated as a cause for Barr-body loss. Chaligné et al. (2015) have shown that breast tumours and cell lines frequently display major epigenetic instability of the inactive X chromosome.

The French researchers also demonstrated that many of genes involved in cancer promotion are aberrantly reactivated in primary breast tumours, and that epigenetic instability of the inactive X chromosome can lead to perturbed dosage of X-linked factors. Taken together, the study provided the first integrated analysis of the inactive X chromosome in the context of breast cancer and established that epigenetic erosion of the inactive X can lead to the disappearance of the Barr body in breast cancers. This research work opened up the possibility of exploiting the inactive X chromosome as an epigenetic biomarker at the molecular and cytological levels in breast cancer, and may be in other types of cancer (Chaligné et. al., 2015).

Australian researchers, mainly from The University of Melbourne and Monash University, Clayton, Victoria, Cancer Council Victoria, and the Hunstman Cancer Institute, Salt Lake City, Utah, United States, published in the 28 February 2018 issue of *Nature Communications* their results on the heritable DNA methylation marks associated with susceptibility to breast cancer. They aimed to scan 480,000 genomic sites for heritable methylation marks associated with breast cancer susceptibility by examining the blood cells of 210 women belonging to 25 Australian multiple-case breast cancer families; among these 210 women 87 had a breast cancer. They reported genome-wide DNA methylation measured in the 210 peripheral blood DNA samples using the Infinium Human Methylation450. They developed and applied a new statistical method to identify heritable methylation marks. They identified 24 methylation marks with corresponding carrier probabilities significantly associated with breast cancer. They replicated an association with breast-cancer risk for four of the 24 marks using an independent nested case-control study (Joo et al., Southey, M.C., 2018).

It has been emphasized that these methylation sites corresponded to four genes different from BRCA genes. Among them “the gene GREB1 was identified and the activity of this gene is controlled by estrogens, associated with breast cancers,” stated Dominique Stoppa-Lyonnet, at that time head of the genetics department at the Curie Institute and professor at the University Paris-Descartes. Cancer risk was increased by 18% to 26%, while a mutation of a BRCA gene increased that risk ten or 20-fold times! (Rosier, 2018)

While both Olivier Caron and Dominique Stoppa-Lyonnet stated that we were rather far from significantly improving the test indicating a genetic susceptibility to breast cancer, “the potential of this approach may be extremely important for cancer diagnosis,” stated Deborah Bourc of the Curie Institute. “It would be possible to analyze the methylation profiles of the genomes of

tumoural cells circulating in the bloodstream and to find out the organ where the tumour exists, without making a biopsy," she added (Rosier, 2018).

Despite the major weakness of the study – i.e. two-thirds of examined women, within the families at risk, received chemotherapy that might have altered the epigenetic sites of their genome – the interest of the research work "is that genetic sites could be modified, much more easily than trying to correct mutations; it could therefore be possible to wake up antitumour genes, or conversely, switch off oncogenes," stated Edith Heard. Melissa Southey, of the University of Melbourne, Victoria, and the School of Clinical Sciences at Monash University, Clayton, Victoria, Australia, *on her side*, emphasized that "our work opens the way to developing epigenetic drugs against breast cancer." In fact there are already drugs that target epigenetic processes and two of them were marketed in France and used against blood cancers. At the international congress on targeted therapies against cancers, held in Paris on 5 March 2018, Antoine Italiano, an oncologist at the Bergonié Institute in Bordeaux, has presented a clinical trial aiming at evaluating the efficiency of a new class of epigenetic molecules against lymphomas and rare solid tumours. It could therefore be predicted that this research may lead to both detecting certain forms of cancer and trying to cure them (Rosier, 2018).

Cancer immunotherapy

At the congress of the American Society of Clinical Oncology (ASCO) – the world's largest cancer conference – held in Chicago in June 2013, with 35,000 specialists in attendance, immunotherapy was the main focus of the meeting (Pollack, 2014). The 2018 Nobel Prize in Physiology or Medicine has been awarded to two pioneers and discoverers of treating cancer by immunotherapy: James Allison, a United States citizen born in 1948 and Tasuku Honjo, a Japanese citizen born in 1942. In 2014 both immunologists were jointly awarded the Tang Prize, an Asian version of the Nobel Prize. Born on 27 January 1942 in Kyoto, Tasuku Honjo was graduated in 1975 from the University of Kyoto, with a Ph.D thesis in medical chemistry. Between 1971 and 1974 he has been invited as guest researcher at Washington's Carnegie Institution and the National Institutes of Health, Bethesda, Maryland. After holding professorships in Tokyo and Osaka, he has been since 1984 associated with the University of Kyoto, of which he has become deputy director-general since 2017. James Allison was born on 7 August 1948 in Alice, Texas: after having passed a PhD in 1973 in Austin, he specialized in cancer immunology at the University of California, Berkeley, and thereafter he pursued his career in New York. He is now affiliated with Texas University in Houston and with the Parker Institute for Cancer Immunotherapy in San Francisco (Cabut, 2018).

Initial research work and its development

The immune system mounts the body's defense and offense against unwanted intrusions: viruses, bacteria and even cancer cells. However malignant cells develop from normal cells that start to grow out of control, and the immune system is specifically programmed not to attack the body's own cells. But J. Allison and T. Honjo, working independently, have found ways to retrain the body to recognize and destroy tumour cells. Their research work began at MD Anderson Cancer Center, Houston, and Kyoto University in the 1990s, and they discovered different ways in which the immune system is blocked from attacking tumour cells. In other words their strategy was to unleash the capacity of the immune system to attack tumour cells thanks to a new class of drugs, called *checkpoint inhibitors*, that allow the immune system to see cancer cells and attack them, drastically improving remission rates. In the past five years (2013-2018) the Food and Drug Administration (US FDA) has approved a dozen new cancer drugs and therapies that boost the immune system in order to make it able to attack tumour cells (Park, 2018).

During the 1990s in his laboratory at the University of California, Berkeley, J. Allison was among the first researchers to discover a protein of T-lymphocytes, called CTLA-4, which was an inhibitor of lymphocytes to attack cancer cells. J. Allison developed an antibody that would bind to the receptor CTLA-4 and thus block its inhibition function. As of 1994 he tested this approach using mice with solid tumours for his experiments. The results obtained were considered outstanding. "But, despite the lack of interest from the pharmaceutical industry, J. Allison carried out many experiments in order to apply this treatment to humans," as stated by the Nobel Committee who selected him as the 2018 Nobel Prize winner. In 2004 J. Allison left the University of California, Berkeley, and went to pursue his research at the Memorial Sloan-Kettering Cancer Research Center in New York. He could there participate in the first clinical trials involving patients with metastatic melanoma. The positive results obtained with a monoclonal antibody called ipilimumab, were confirmed on a larger scale, in the treatment of melanoma, and thereafter of other cancers (lung, prostate, ovary and pancreas). "It should be underlined that J. Allison was the first to apply this approach in humans, at a time when the majority of scientists in the medical community had serious doubts about the feasibility and effectiveness of such a strategy," commented the Balzan Foundation which rewarded the researcher in 2017, two years after the Lasker Prize, awarded to researchers in the biomedical sciences. *The monoclonal antibody ipilimumab (Yervoy, anti-CTLA-4) was the first checkpoint inhibitor approved by the FDA in the United States and in Europe in 2011* (Cabut, 2018).

Also in the early 1990s Tasuku Honjo, on his side, had discovered another protein expressed on the surface of T-lymphocytes, called PD-1 (programmed death receptor 1). He also carried out a number of experiments in his University of Kyoto laboratory, which led to the conclusion that PD-1 – is the same way as CTLA-4 – blocks the action of T-lymphocytes, but through a different mechanism. Since then *many monoclonal antibodies anti-PD-1* have been developed and two of them have been commercialized, *nivolumab and pembrolizumab*. Tasuku Honjo's and followers' research work also led to the development of antibodies anti-PD-L1, another protein present on the surface of tumoural cells, that binds to the monoclonal antibodies (Cabut, 2018).

Skin melanoma

Skin melanoma is a malignant tumour of melanocytes – the cells which give the skin its colour. In France, for instance, new cases of skin melanoma amount to 14,000 annually – which makes it the ninth most occurring cancer in this country. In a large number of cases the prognosis of skin melanoma is generally favourable. The tumour is removed surgically and the patients are monitored by their dermatologist and their general practitioner. But *ca*. 20% of primitive skin melanomas will become metastatic. This happens in particular in the case of so-called "thick" melanomas, of which the initial surgical treatment includes the identification of a tumour-draining node, called "sentinel node". This kind of melanoma must be closely monitored and in order to prevent recurrence after surgery, "adjuvant treatments" are carried out and they have received their authorization at the European level (Mongis, 2018).

But before giving more details on these "adjuvant" treatments, it should be mentioned that a therapeutic revolution over a decade has drastically changed the treatment of patients suffering from metastatic melanoma. Firstly, the research for a mutation called BRAF and present in 50% of metastatic melanomas leads to targeted therapies combining a BRAF inhibitor and of another enzyme, MEK. In France two sets of targeted therapies anti-BRAF MEK are available: the combination of vemurafenib-cobimetinib and that of dabrafenib-tetrametinib. A third combination eucorafenib-binimetinib has been authorized at the European level. With these targeted therapies, the effect is extremely fast and important. *Ca*. three-quarters of patients receiving targeted therapies

develop a resistance after three years and therefore they need other treatments. But others show a durable response as well as a quite acceptable tolerance of the treatment (Mongis, 2018).

Targeted therapies a well as anti-PD-1 therapies have been authorized in Europe in the preventive treatment, called "adjuvant", of recurrence, in the case of patients with node (ganglion) affection; they reduce by *ca*. 50% the recurrence of the disease. Targeted therapies have side effects, which are not too serious and which generally disappear (Mongis, 2018). Another tool against metastatic melanoma is that of immune therapies.

Melanoma and immunotherapies

The new class of drugs or checkpoint inhibitors "have changed the life of our patients suffering from metastatic melanoma," stated Caroline Robert, the head of dermatology department at the French Gustave-Roussy Institute, Villejuif, south of Paris, who was among the first practitioners to test the effect of checkpoint inhibitors among patients with metastatic melanoma. "Firstly, there was the monoclonal antibody ipilimumab (anti-CTLA-4) which triggered a response in 15% of patients who had tried other unsuccessful treatments. With the anti-PD1, the response rate increased up to 40%. And some of the patients are still in complete remission with more than five years without a relapse, and even ten years for those patients who had been the first to be treated with ipilimumab." Even though physicians are reluctant to consider that these patients are cured, they are indeed, whereas they were initially diagnosed to have a very short life expectancy (Cabut, 2018).

On Thursday 4 September 2014 the US Food and Drug Administration (FDA) granted accelerated approval to pembrolizumab (Keytruda, Merck Sharp & Dohme Corp.) for the treatment of patients with unresectable or metastatic melanoma and disease progression following ipilimumab. Pembrolizumab is a monoclonal antibody that binds to the PD-1 receptor and blocks its interaction with PD-L1 and PD-L2, thereby releasing PD-1 pathway-mediated inhibition of the immune response, including antitumour immune response. As a condition of this accelerated approval, Merck was required to conduct a multicenter, randomized trial establishing the superiority of pembrolizumab over standard therapy to verify and describe the clinical benefit of this monoclonal antibody. The recommended dose of pembrolizumab is 2mg/kg administered as an intravenous infusion over 30 minutes every three weeks.

Keytruda was the sixth new antimelanoma drug approved since 2011, transforming the care of a disease that, once it had spread, usually meant a quick death. The accelerated approval granted to Keytruda allowed Merck to win a race to market its product in the United States, against Bristol-Myers Squibb (BMS), Roche and AstraZeneca, which were also in advanced stages of testing drugs that also block the action of PD-1 (Pollack, 2014; Sasson, 2016).

Keytruda was approved after, what was essentially an extra-large phase-1 trial involving 173 participants who all received the drug, with no control group. Tumours shrank in *ca*. 24% of patients, the FDA stated, with the therapeutic effect lasting at least 1.4 to 8.5 months and continuing beyond this period in most patients. "Even the very preliminary results on a handful of patients indicated a high degree of activity," stated Richard Pazdur, who oversaw cancer drugs at the FDA, in an interview on 4 September 2014 (Pollack, 2014).

Lung cancer and immunotherapy

Researchers found that the anti-PD-1 drugs worked for some patients with lung cancer. This cancer is the leading cause of cancer death globally, *ca*. 1.7 million deaths a year. In the United States it was expected to kill more than 154,000 people in 2018. "If you want to use long-term

survival, you've got to give immunotherapy as soon as possible," stated Roy Herbert, chief of medical oncology at the Yale Cancer Center. "Chemotherapy has limitations. Immunotherapy has the ability to cure. I lead the Yale lung team. We have patients on these immunotherapies alive for more than eight years," he added (Grady, 2018).

At a meeting of the American Association of Cancer Research, held in Chicago in April 2018, Leena Gandhi, director of the Thoracic Medical Oncology Program at the Perlmutter Cancer Center at New York University Health, presented the results of a study including 616 patients with an advanced stage of non-squamous non-small-cell lung cancer, from medical centres in 16 countries. They were picked at random to receive either chemotherapy plus immunotherapy, or chemotherapy plus a placebo, with two-thirds receiving the combination that included immunotherapy. The immune-activating drug was Keytruda, the chemotherapeutic drug is called pemetrexed, plus either carboplatin or cisplatin. After a median follow-up of 10.5 months, those in the immunotherapy group were half as likely to die. The median overall survival was 11.3 months in those who did not receive immunotherapy, whereas survival in the immunotherapy group was longer and the median has not yet been reached. But patients in the immunotherapy group had more kidney problems, more immune-related adverse events and were more likely to stop treatment because of these side-effects. The estimated survival at 12 months was 69.2% in the group that received immunotherapy, and 49.4% in those who did not. These results were published in *The New England Journal of Medicine* (Gandhi et al., and Garassino, 2018).

Other studies in lung cancer have involved another anti-PD-1 monoclonal antibody, nivolumab (or Opdivo, made by Bristol-Myers Squibb), which works in a similar way to psembrolizunab. According to Roy Herbst of the Yale Cancer Center, most patients treated stayed on the drugs for two years. One Yale patient who has survived for eight years took the drug for two years and has remained well ever since. Another had to stop because of side effects after only two or three months, but was still well two years later. R. Herbst offered an explanation about why chemotherapy and immunotherapy could work well together. He said that tumour cells were like bags of proteins that the immune system could use as targets to find and thus attack cancer. By killing some tumour cells, chemotherapy could open the bags and help the immune cells – unleashed by the anti-PD-1 drugs – to identify their target. It is also possible, he stated, that chemotherapy may kill some immune cells that interfere with the cancer-killing action of other parts of the immune system. R. Herbst concluded: "We are making progress, but sill only benefiting 30% to 40% of patients. There is a lot more room to do better" (Grady, 2018; Herbst et al., 2018; Doroshow and Herbst, 2018).

This is precisely what did Merck & Co. who revealed on 3 June 2018 a later study of almost 1,300 patients that showed that its immunotherapy drug, Keytruda, boosted survival in patients with an advanced stage of lung cancer. Patients taking Keytruda lived for an average of 16.7 months versus 12.1 months on chemotherapy. Those whose cancers had a very high level of PD-1 survived for 20 months on average when taking Keytruda – almost twice as long as those on chemotherapy. These data were presented at the annual meeting of the American Society of Clinical Oncology in Chicago, where physicians hailed immunotherapies as the biggest advance in tackling the disease for decades. Merck & Co cemented its position as the dominant developer of immunotherapy drugs, when physicians and experts have described the results from the combination of chemo- and immunotherapy as "practice changing", putting the immunotherapy and chemotherapy cocktail to become the standard treatment for the vast majority of cancer sufferers (Crow, 2018).

Roger Perlmutter, Merck's senior scientist, agreed, pointing out that medical doctors would prefer not to use chemotherapy on some patients, such as the elderly or people with illnesses besides

cancer. On the market place, drug makers including Merck, Bristol-Myers and Squibb, Roche and AstraZeneca have generated in 2017 almost US$10 billion in sales. But the market for untreated lung cancer patients, where Merck now dominates, is the most lucrative because it is among the most prevalent forms of the disease as well as being the deadliest (Crow, 2018).

Breast-cancer treatment: combining immunotherapy with conventional therapies

"All cancer patients will likely receive (immunotherapy) in five years, so it is going to be curative for a lot of them," predicted James Allison by 2017-2018. Immunotherapy treatments are especially effective against lung cancer, skin cancer and blood cancers like leukemia and lymphoma. But immune-based treatments have not been as successful, or as plentiful, for the most common cancers: colon, prostate and especially breast. Of the more of than 600,000 (*ca*. 620,000) people who died of cancer in the United States in 2017, the vast majority had these types of solid tumours. According to Robert Vonderheide, director of the Abramson Cancer Center at the University of Pennsylvania, "most breast cancers fit into a category called 'cold' immunological tumours, meaning that the tumour has the ability to either exclude the immune system or hide from it all together. That kind of cancer is not easily treated with current immunotherapies." At least not yet. Building on the foundation that J. Allison and T. Honjo established with checkpoint inhibitors, researchers are finding curative ways to boost the immune system to see tumours like breast cancer are "hot" rather than "cold" targets, in the same way infectious-disease pathogens like measles or influenza viruses flag a response (Park, 2018). In 2018 there was not yet an immune-based treatment approved in the United States specifically for breast cancer.

While chemotherapy and radiation in their current forms destroy immune cells along with malignant ones, modified formulas may be just enough to stimulate an inflammatory response that awakens the immune system to see and attack tumour cells. Breast-cancer researchers are testing ways to then deploy immunotherapy drugs like checkpoint inhibitors to elicit the strongest immune response against tumours. Steven Rosenberg, chief of surgery at the National Cancer Institute, studies the mutations that drive his patients' breast cancer, isolates the few immune cells that are trying to fight the cancerous cells, amplifies their numbers in the laboratory and injects them black into his patients. He believes that this approach will have the same success immunotherapies have had in blood, lung and skin cancers, with regard to the more common malignancies in the breast, prostate and colon (Park, 2018).

In the 1980s S. Rosenberg became among the first to notice that some mutations also attract the attention of the immune system, and some immune cells can start infiltrating the tumours. At the time, S. Rosenberg stated: "Nobody knew there was an immune response against human cancers." He decided to isolate T-cells from 195 people with melanoma, expanding their numbers and infusing them back to the patients. So far, 30% of them have responded completely to the therapy, meaning their existing cancer cells disappeared and they have not seen any tumours reappear in nearly seven years since the beginning of treatment (Park, 2018).

Leukemia and lymphomas are particularly amenable to a type of immune therapy that involves replacing patients' malignant blood with a population of their T-cells that are processed to attack a receptor common among cancerous blood cells. Up to 90% of people with certain types of leukemia, whose cancer recurred after repeated cycles of conventional treatments, have gone into remission after receiving this form of immunotherapy. This success led the FDA to approve immune-cell-based therapy, called CART, for a type of leukemia and other blood cancers in 2017 (see below) [Park, 2018].

But solid cancers – which are much more common – have fewer mutations, and the tissues they invade (like those in the breast) cannot be replaced as blood cells can, making immunotherapy more difficult. Robert Vonderheide has found that of the 7,000 tumours listed in a national genetic database, breast cancers fell in the bottom 25% of tumours when it came to how many mutations they carried. Because of that, breast cancers are also part of the bottom half of all cancers when it comes the immune responses the body generates again them. "Breast cancer notoriously shields itself from the immune system," he stated (Park, 2018)

The first breast-cancer patient in S. Rosenberg's study had stage IV cancer that had recurred and spread to lumps in her chest and to her liver, despite a dozen chemotherapy and hormone treatments and even a mastectomy. S. Rosenberg did a thorough analysis of her tumour and found 62 major mutations responsible for turning the patient's cells malignant. He then searched for few immune cells that could recognize and attack four of those genetic aberrations and were already battling her cancer. He extracted those immune cells, grew them in large numbers in the laboratory and returned them to the patient as an immune-based treatment against her breast cancer. Within a month of receiving the onetime infusion of cells, she felt the tumour in her chest become "softer and smaller". Within two months, the tennis-ball-size growth in her liver had disappeared and the tumour in her chest had also shrunk to nothing. Nearly three years later, physicians said she was in a durable regression. But in 2018 only 14% of the 42 people S. Rosenberg has treated have responded as the first patient had. He believes that this percentage will increase if he and others find better ways to pinpoint both the mutations behind each patient's cancer and the populations of immune cells targeted against them (Park, 2018).

At the Abramson Cancer Center at the University of Pennsylvania, Robert Vonderheide is experimenting with ways to combine newer immunotherapy drugs with conventional treatments like chemotherapy and radiation with the hope that synergistic effect will make tumours more visible and vulnerable to immune attack. James Allison, the Nobel Prize Laureate, stated that the idea is to "turn radiation and chemotherapy into a sort of vaccine." The key is not using standard cycles of chemo or radiation, but tweaking the treatments so they are just right for activating an immune response. Too much chemotherapy or radiation suppresses the immune system, but just enough can act like a stimulant to activate it. Peter Schmid, clinical director of the breast-cancer center at the St. Bartholomew Cancer Center in London, was to announce much anticipated results at the end of October 2018 from a study that combines a chemotherapy agent with a checkpoint inhibitor for treating advanced triple-negative breast cancer, an aggressive, difficult-to-treat form of the disease. The chemotherapy agent is delivered in nanoparticle form, which makes it more soluble and better equipped to slip inside cell membranes to activate an immune response (Park, 2018).

There are similar expectations over combining shorter schedules of radiation with checkpoint inhibitors. This approach shows even more promise as a way to target tumours that have spread to hard-to-reach tissues – a common issue with breast cancer. Researchers believe that it is because radiation given over a few days rather than over the standard week-long schedule may be enough to trigger an immune response against a specific tumour, which is then directed to attack tumour cells in other parts of the body. In the case of breast cancer, researchers hope that this response will find growths that have spread beyond the breast and target those. This new thinking is "really disruptive," stated J. Allison, "because we are realizing with chemotherapy and radiation that we do not need to kill every last tumour cell, but stir up things just enough for the immune system to take them out" (Park, 2018).

Studies like the one in which an American patient, Kathy James, is participating in the United States, could also overturn the current thinking about how to treat one of the stubborn challenges

of breast cancer-recurring tumours. If vaccines designed to awaken immune cells against cancer are effective, then breast-cancer patients could potentially be protected from having their cancer return with periodic anticancer "booster" shots. Their immune systems would mainly be primed to find and eliminate any cancer cells before they can coalesce into tumours (Park, 2018).

Chimeric antigen receptor T-lymphocytes (CART-cells)

As mentioned previously there have been very good clinical results, when applying a cell therapy combined with a gene therapy to the treatment of acute lymphoblastic leukemia on non-Hodgkin malignant lymphomas at an advanced stage. The patients received their own T-lymphocytes (autologous) that had been genetically modified in the laboratory before being administered. After this genetic modification the T-lymphocytes express a chimeric antigen receptor, or CAR, that can recognize a protein expressed on the surface of tumour cells. The genetically modified CART-cells behave like searching "missiles" that recognize the protein target expressed on cancer cells and thereafter destroy them. Despite some side-effects –sometimes severe – seen in some patients, this cell therapy has attracted pharmaceutical companies: e.g. Novartis, GlaxoSmithKline (GSK), Pfizeer, Janssen, Amgen or Celgene invest big amounts of money in clinical trials of CART-cells, and more than 20 biotechnology companies, such as Juno, Kite, Pharma, Bluebird Bio or Cellectis (a French biotech), seize this opportunity to highlight the benefit of their genetic-engineering technologies (Chabannon et al., 2015; see Sasson, 2016).

Cellectis success story

The biotechnology company Cellectis started in the late 1990s when André Choulika, co-founder and executive officer of the company, was working at the Pasteur Institute in Paris and discovered molecular tools (meganucleases) or DNA "scissors" that can modify specific DNA sequences and thereby transform the cell's genome. In 1999 he founded Cellectis with another researcher of the Pasteur Institute, David Sourdive. Ten years later they were the first to sell "ready-to-work" kits which made that technology available to cell-biology researchers, with the aim to engineering human cells (see Sasson, 2016).

In January 2014 A. Choulika attended the JP Morgan Healthcare Conference in San Francisco, and he brought with him several scientific papers on a new biotechnology tool: genetically modified T-lymphocytes that could target tumour cells and kill them. Those so-called CART immune cells were the research focus of several teams in the United States, since Carl June, a researcher at the University of Pennsylvania, had successfully used them in a patient suffering from leukemia. In June 2014 Cellectis made a historical deal with the big pharma Pfizer which owned 10% of Cellectis equity. Thanks to Pfizer, Cellectis had more than €100 million in its cash flow and could therefore fund its own research projects, in addition to those carried out in collaboration with the big pharma (see Sasson, 2016).

"The approach consisting of engineer T-lymphocytes and reinject them into the same patient was very costly, from US$500,000 to US$1 million per patient and the process cannot be scaled up," stated A. Choulika. On 5 November 2015 Cellectis announced that one of its experimental immune-treatments of cancer had been successful, when used to treat an 11- month-old British child, suffering from leukemia that was resistant to all existing conventional treatments. The very young girl, Layla Richards, had been hospitalized at London's Great Ormond Street Hospital (GOSH) in the Bone Marrow Transplant Unit led by Paul Veys (see Sasson, 2016).

Only 200 patients worldwide have benefited from this kind of immunotherapy until November 2015. This was due to the fact that currently CART-cells are engineered from the patient's own

immune cells so as to avoid their rejection afterwards. Each dose of transformed cells being unique, the production is done at a very small scale and consequently the cost of the treatment is very high. Since this treatment requires a customized approach, it cannot be mass-produced as a universal, off-the-shelf procedure for any patient. A bioindustrial production scheme has nevertheless to be set up in order to apply this efficient immunotherapy to the large number of patients put on waiting lists (see Sasson, 2016).

Rival companies, such as Juno, founded by the end of 2013, and Kite which was listed on the Nasdaq in June 2014, had more funds or stock exchange value than Cellectis. Both companies are scientifically and technologically at the same stage of advancement in their research work as Cellectis, which may catch up in terms of funding (one should note that by early 2014 Cellectis was almost bankrupt) A. Choulika considered that if the approach chosen is efficient, it will be possible to standardize the manufacture of drugs and to commercialize them at an affordable cost. After the British infant had received in 2015 a treatment called "UCART19", exceptionally, clinical trials were initiated at the end of 2015 with 12 British patients; commercialization of this immunotherapy was not foreseen before at least 2020. Thanks to the very positive results obtained, Cellectis listed in Paris and New York stock exchanges, was valued in 2015 at US$1.4 billion, behind its rivals, Kite (US$3.1 billion) and Juno (US$ 5.1 billion) [see Sasson, 2016]

Prospects of immunotherapy in combating cancers

The immunotherapeutic compound was commercialized in 2011: this was Bristol-Myers Squibb (BMS) Yervoy (ipilimumab), the sales of which amounted to US$1.4 billion in 2014. Two other molecules were approved by the end of 2014 in the United States and at the beginning of 2015 in Europe: Opdivo (nivolumab), also made by BMS, and Keytruda (pembrolizumab), made by Merck & Co. These drugs were initially used in the treatment of advanced melanomas, but they also were exceptionally effective in the treatment of some lung cancers, much more common than melanomas. The market of these probable blockbuster drugs was estimated at US$33 billion by 2022. Competition is rising among pharmaceutical groups, including Roche, AstraZeneca and Sanofi, in order to develop new immuno-oncological drugs (cf. Sasson, 2016).

Researchers had observed that only 25% to 30% of patients reacted to the already commercialized antibodies and that a promising approach was to use a combination of several molecules. That was the purpose of the deal between BMS and Innate Pharma, a Marseille-based biotechnology firm (south-east of France). This deal opened up the American market to the French company. In 2011 the startup initiated its first road show in the United States and it was a real success: in November 2011 it was able to raise in one day €24 million. In 2015, 40% of the company's equity belonged to American investors, including Orbimed – one of the most renowned hedge funds in the health-care sector (cf. Sasson, 2016). According to Martial Descoutures, a health-care analyst at Invest Securities, "immunotherapeutic treatments are expected to become the cornerstone of cancer treatment, and consequently companies such as BMS and AstraZeneca (and others) are seeking to find out and secure the most effective compounds and combinations of the latter" (cf. Sasson, 2016).

The CART-cells technique also holds great promise, especially when it could be improved biotechnologically and scaled up to meet the needs of a large number of cancer patients.

Access to expensive cancer immunotherapies

Coming back to Keytruda (pembrolizumab) which has become available to cancer patients in France, as a first line of treatment, its cost has been evaluated at *ca*. € 6,000 per month and per

patient, much higher than conventional chemotherapy treatments. On 28 November 2017 this cost, to be reimbursed by the French Disease Insurance, was negotiated after several months of discussion between the French social security services and the MSD laboratory, a subsidiary of Merck & Co. The treatment with Keytruda has shown an improvement of survival of three to four years in the case of non-small-cell metastatic lung cancers and the tumours of which express the PD-1 biomarker, i.e. *ca*. 20% of the 45,000 patients suffering from lung cancer each year in France. This equals to 6,000 to 8,000 patients. According to Christos Chouaid, a lung-disease specialist at the University Hospital in Créteil, south of Paris, involved in the clinical trials, "the results are spectacular"; "the survival rate has more than doubled thanks to Keytruda, compared with chemotherapy, and with better tolerance," added Nassima Mimoun, an oncology senior scientist at MSD (Santi, 2017). Keytruda had been allowed to reach the market by the end of 2014 in France, thereafter in January 2017 in the European Union, and it took about a year to agree on the cost of the drug. Experts considered that the delay was too long. Because the patients that can receive this treatment should quickly benefit from it, stated on 23 November 2017 at a press conference Aurelien Marabella, director of the immunotherapy programme at the Gustave-Roussy Institute (IGR, Villejuif, south of Paris) and member of the Reflexion Circle in Immuno-oncology (CRI, French acronym) [Santi, 2017].

Ca. more than 1,000 clinical trials of immunotherapy against cancers were being carried out at the beginning of 2018; the vast majority of these trials were focused on the efficiency of combinations of already available drugs. At the Gustave-Roussy Institute, such combinations amounted to 50% of treatments. Although they are sometimes hailed as "miracle" drugs, these immunotherapy drugs have shown their efficiency in clinical trials – and depending on the kind of tumours – among 10% to 40% of patients (Santi, 2017).

However, immune checkpoint inhibitors (ICIs) can also cause severe or fatal immune-related adverse-events (irAEs). American and French researchers, with funding from the US National Cancer Institute (National Institutes of Health, NIH), the James C. Bradford Jr. Melanoma Fund and the Melanoma Research Foundation, on the one hand, and the Cancer Institute thématique multi-organismes of the French National Alliance for Life and Health Sciences, Plan Cancer 2014-2019, on the other, aimed to identify and characterize cardiovascular irAEs that are significantly associated with ICIs (Salem et al., 2018).

In this observational, retrospective, pharmacovigilance study, they used VigiBase, World Health Organization's (WHO) global database of individual case safety reports, to compare cardiovascular adverse event reporting in patients who received ICIs with this reporting in the full database. The study was carried out between 14 November 1967 and 2 January 2018. The researchers identified 31,321 adverse events reported in patients who received ICIs and 16,343,451 adverse events exported in patients treated with any drugs (full database) in VigiBase. Compared with the full database, ICI treatment was associated with higher reporting of myocarditis, pericardial diseases and vasculitis, including temporal arteritis and polymyalgia rheumatica. The researchers concluded that treatment with ICIs can lead to severe and disabling inflammatory cardiovascular irAEs soon after the beginning of therapy. In addition to life-threatening myocarditis, these toxicities include pericardial diseases and temporal arteritis with a risk of blindness. These events should be considered in patient care and in combination clinical trial designs (i.e., combinations of different immunotherapies as well as immunotherapies and chemotherapy) [Salem et al., 2018].

But, more importantly, not only these treatments are delivered on the market with lengthy delays, but they are too expensive; henceforth the long negotiations with the public insurance system or with private insurers. According to Jean-Yves Blay, an oncologist specialized in the

treatment of sarcomas, "the delays and constraints relating to the cost limitation of these drugs hamper innovation, while the reimbursement of old treatments is not questioned; particularly of those whose efficacy is not established, such as homeopathic drugs." In France, drugs should be evaluated by the Health High Authority, thereafter by the Economic Committee for Health Products (CEPS, French acronym), which establishes their cost, often very high. Such process can last for months. On the other hand, the bodies in charge of evaluating and authorizing clinical trials are overwhelmed and lack the necessary tools to be efficient. It was observed that "if regulatory obstacles were to last, France would become less attractive (Nassima Mimoun)," while this country was regarded as particularly active in oncology (Santi, 2017).

Again in France, as an example, the representatives of patients express their unsatisfaction. For instance, the French Association of Multiple Myeloma Diseases has strongly complained on 25 November 2017 about the "unacceptable delays" for authorizing five immunotherapy drugs that had obtained their market authorization in Europe by the end of 2015, and were not yet available in France. With respect to childhood cancers, there are organizations who are struggling to lay more emphasis on oncological research and are deploring unequalities in the access to clinical trials, due to the lack of information (Santi, 2017).

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THE RISING WORLD OF MICRORNAS: ONCOGENES AND TUMOR SUPPRESSORS IN CANCER

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Abstract

The sequencing of the human genome has revealed that the bulk of the genome is transcribed into noncoding RNAs, including microRNAs. These microRNAs are deregulated in cancer where they regulate a wide array of functions associated with tumorigenesis. Glioblastoma (GBM) is the most common and aggressive primary intracranial tumor of the adult brain. miRNAs are short noncoding RNAs that broadly regulate gene expression. They exert their actions via transcriptional, post-transcriptional, and epigenetic mechanisms that are poorly understood. Numerous miRNAs are deregulated in GBM, where their expression levels can serve as biomarkers. They can act as either oncogenes or tumor suppressors in GBM by targeting the expression of numerous tumor-suppressors or oncogenes. miRNAs regulate all GBM malignancy aspects including cell proliferation, survival, motility, migration, invasion, angiogenesis, cancer stem cell biology, microenvironment modulation, immune escape, and therapy resistance. MiRNAs can also be secreted via exosomes or microvesicles in body fluids, where they can be used as diagnosis and/or prognosis biomarkers. Considering their deep involvement in GBM malignancy, numerous studies are currently undertaken to exploit miRNAs as therapeutic agents or targets. This short review summarizes the biogenesis, deregulation, functions, mechanisms of action, and clinical applications of miRNAs in GBM.

Key words

Glioblastoma, glioma, microRNA, glioma stem cells, drug resistance.

Conflicts of interest: The authors declare that they have no conflict of interest.

Introduction

More than 80% of the genome is transcribed into RNA, but only 2-2.5% is translated into proteins. Therefore, the vast majority of the RNAs are noncoding RNAs (lncRNAs; >200 nucleotides), proven to be key regulators of different physiological and pathological processes overriding the previous paradigm that they are evolutionary junk.

In the last decade, the small ncRNAs (microRNAs; 17–22 nucleotides) and long ncRNAs have been extensively studied in different pathologies including cancer, have improved our understanding of cancer initiation and progression, and paved the way for new therapeutic strategies. Computational predictions of miRNA targets suggest that more than 60% of human protein expressions are regulated by miRNAs and thus have critical functions across various biological processes. Notably, single miRNAs can regulate the expression of numerous genes and most genes are regulated by multiple miRNAs. A large number of studies have shown that microRNAs play important roles in almost every aspect of cancer, including tumor initiation, progression, and resistance to therapy, as well as providing biomarkers for diagnosis and prognosis and serving as therapeutic agents or targets. This short review summarizes the roles of microRNAs in glioblastoma (GBM).

Glioblastoma

Glioblastoma (GBM), also known as grade IV astrocytoma, is the most aggressive primary intracranial tumor of the adult brain. Gliomas are extremely aggressive tumors that account for the majority of deaths due to primary brain neoplasms (1). Despite the most advanced treatment that combines surgery, radiotherapy, and chemotherapy, the most commonly diagnosed grade IV GBM is often associated with a poor survival reaching a life expectancy of only 14 months, in the best prognosis. The etiology of GBM is largely unknown, but the literature has already established that GBM malignancy is tightly dependent on the deregulation of pathways and molecules that control cell proliferation, survival, apoptosis, migration, invasion, angiogenesis, and stem cell differentiation (2). According to the cancer Genome Atlas, these deregulations were divided into 3 canonical pathways: p53, Rb and receptor tyrosine kinase (RTK) pathways (3). GBMs' poor prognosis is a consequence of rapid cell proliferation, cell survival, inhibition of apoptosis, cell migration, fast invasion of surrounding brain tissues, extensive angiogenesis, evasion of the immune system, and the presence of Glioma stem cells (GSCs) niches that sustain resistance to chemotherapy and radiotherapy.

MicroRNAs

Non-coding RNAs are RNA molecules that are not translated into proteins but are nevertheless functional. They are known to regulate gene expression at both transcriptional and post-transcriptional levels. Of all non-coding RNA species, microRNAs are the best characterized in terms of biogenesis and functions.

MicroRNAs (miRNAs) are a class of small highly-conserved non-coding RNA species (17–25 nucleotides) that post-transcriptionally regulate gene expression. In terms of biogenesis, one-third of miRNAs are transcribed as single transcriptional units or in clusters (4-7) as pri-miRNA by RNA polymerase II and then excised into pre-miRNA by the RNase III enzyme called Drosha. The pre-miRNA is subsequently exported to the cytoplasm by exportyin 5, and processed into a mature complex by the Dicer complex (8-10). The mature thermodynamically stable single strand miRNA is then incorporated in the RNA-induced silencing complex (RISC) and directed to the mRNA 3′ untranslated region (3′UTR) (11). Depending on the degree of complementarities with their targets' 3′ UTR, miRNAs directly cleave the mRNA or inhibit protein synthesis (Figure 1) (12).

MiRNAs function as tumor suppressors or oncogenes by targeting oncogenic proteins or tumor-suppressor proteins, respectively. Like in other cancers, miRNAs regulate all aspects of glioma malignancy and progression including cell cycle, cell proliferation, cell death, survival, apoptosis, migration, invasion, metastasis, angiogenesis, tumor microenvironment modulation, tumor immune response, and glioma stem cell biology (4). A great deal of progress has been

made recently in dissecting miRNA pathways associated with cancer initiation, progression, and metastasis (13, 14).

MicroRNA expression, called gene signatures, also contributes to the phenotypic diversity of GBM subclasses through their ability to regulate developmental growth and differentiation. MiRNAs have been identified as diagnostic and prognostic biomarkers for patient stratification and may also serve as therapeutic targets and agents (15).

Deregulation of microRNAs and its association with GBM clinical features

Recently, seminal studies have reviewed the miRNAs differential expression in GBM and have shown that 95 miRNAs were significantly downregulated and 256 miRNAs were significantly overexpressed in GBM as compared to the normal brain tissue (16, 17).

Historically, miR-21 was the first miRNA reported to be an oncogenic miRNA associated with glioma malignancy. MiR-21 levels are elevated in human glioma cells and tissues as compared to normal glial cells and/or normal brain and correlate with tumor grade (18-20).

MiR-26a has been identified as a key regulator of the tumor suppressor PTEN in gliomas by two prominent studies (21, 22). The first publication showed that miR-26a gene is frequently amplified in human gliomas and that this is associated with monoallelic PTEN loss. Using a multidimensional genomic data set of GBM from TCGA, the second study identified miR-26a as a cooperating component of a frequently occurring amplicon that also contains CDK4 and CENTG1, two oncogenes that regulate the Rb1 and PI3K/Akt pathways, respectively (21, 22).

In our laboratory, analysis of human specimens showed that miR-34a expression is downregulated in GBM tissues compared to normal brain and in mutant p53 gliomas as compared with wild-type p53 gliomas. MiR-34a levels in human gliomas were inversely correlated to the receptor tyrosine kinase cMet, measured in the same tumors (35). MiR-34a was shown to target other oncogenes such as PDGFR, EGFR, Notch1/2 (23).

Another study carried out by our group described miR-148a as an oncogene in GBM. Its expression was elevated in human GBM specimens, cell lines, and GSC compared with normal human brain and astrocytes. High expression of miR-148a significantly correlated with survival in TCGA samples and can serve as a prognostic oncogenic miRNA in GBM (24).

Similarly, miR-10b expression was upregulated in glioma samples as compared to non-neoplastic brain tissues, and its expression levels were associated with higher grade tumors. Several lines of evidence suggest that miR-10b is a key player in glioma invasion (25, 26).

MicroRNAs and stemness

In gliomas, it is well established that GSCs, confer the tumors resistance to chemotherapy and radiotherapy and could be the source of post-radiation tumor recurrence (27). This cellular population was thought to stem from transformed normal stem cells (NSC), a hypothesis that was supported by glioma microRNA profile evocative of neural precursor cells (28). Follows a few examples of the role of microRNA in stemness phenotype in GBMs.

Two studies uncovered critical roles of miRNA-34a in GSCs (23, 29). It was first shown that miR-34a is downregulated in human GBM and exerts potent anti-tumor effects in glioma cells and stem cells via direct inhibition of MET, Notch1, and Notch2 expressions, with Notch being a key regulator of normal and cancer stem cell maintenance (30-32). Notch pathway activation

drives the stemness, proliferation, and radioresistance of GSCs (31-34). These studies underlined the role of miR-34a in the regulation of GSCs partly via regulation of Notch expression.

Another study has shown that the miR-17-92 cluster has been involved in the regulation of GSC differentiation, apoptosis, and proliferation (35). Indeed, expression of several members of miR-17-92 was significantly higher in primary astrocytic tumors than in the normal brain and significantly increased with tumor grade. A high-level amplification of the miR-17-92 locus was also detected in one GBM specimen, while inhibition of miR-17-92 induced apoptosis and decreased cell proliferation of GSCs (35).

MicroRNAs-containing microvesicles as biomarkers in GBM

Tumor antigens' shedding is one of the features of tumor progression. Similar to other tumors, GBM cells shed microvesicles containing abundant amount of microRNAs that can be quantified in human body fluids such as serum and cerebrospinal fluid. MicroRNAs-containing microvesicles or exosomes are used as non-invasive quantitative method of GBM features and are called microRNAs signatures (36, 37). In the case of oncogenic miRNAs, this microvesicles shedding by GBM cells allows them to "share" miRNAs with nearby cells, thereby modifying surrounding stromal cells, and essentially transforming their environment to a pro-tumor context (38).

As GBM signatures, microRNAs released from tumor cells are detected in patient serum and play a critical role in the microenvironment modulation. Many miRNAs have been described as highly expressed in peripheral blood as compared to normal samples (39). MiR-21 is upregulated (40), while miR-205 is downregulated in GBM patients' plasma (41, 42). MiR-454-3p was highly expressed in the plasma of GBM patients as compared to healthy controls and was lower in low-grade glioma. Additionally, miR-454-3p expression in the postoperative plasma is markedly downregulated in comparison to preoperative plasma, and increasing miR-454-3p expression was correlated with poor clinical outcome in gliomas (43).

Different MiR-29 levels in serum can serve to distinguish the progression of GBM malignancy from stage I–II to stage III–IV (44). Similarly, a substantial increase in miR-210 expression was found in serum samples of GBM patients compared to controls and this was associated with tumor grade and poor prognosis (44). A study based on genome-wide miRNA analysis of serum miRNA profiling uncovered a significant difference of miRNA levels between untreated high-grade astrocytomas and controls.

MicroRNAs and immune evasion

The immune system interacts intimately with tumors over the entire process of disease development and progression to metastasis. This complex cross talk between immunity and cancer cells can both inhibit and enhance tumor growth and is now classified as a hallmark of cancer (45), including GBM (46-52).

Numerous miRNAs regulate immune evasion. As examples, miR-124 inhibits STAT3 to enhance T-cell-mediated immune clearance of gliomas (53). Indeed, Treatment of T cells isolated from GBM with miR-124 reversed a block in T-cell proliferation and also reduced expression of STAT3 and forkhead box P3, and subsequently inhibited the development of immune-suppressive regulatory T cells (53). Similarly, using a mouse GBM xenograft models, miR-124 delivery prolonged survival in immuno-competent mice (53).

Another study has shown that Dicer, miR-222, and miR-339 expressions were inversely correlated with the expression of intercellular cell adhesion molecule (ICAM-1) and they enhanced the

susceptibility of tumor cells to antigen-specific lysis by cytotoxic T-lymphocytes. MiR-222 and miR-339 contribute to GBM evasion of the immune system by targeting ICAM-1, which modulates T-cell responses (54).

MicroRNAs and drug resistance

Radio-resistance and chemo-resistance represent a major hurdle in the arsenal of GBM therapy. In terms of response to treatment, several miRNAs have been reported to influence therapeutic sensitivity by interfering with multidrug resistance proteins (MDRP) (55).

MiR-21 influences the efficacy of temozolomide (TMZ) by strongly reducing its effect on apoptosis, through inhibition of pro-apoptotic proteins Bax and caspase-3 as well as upregulation of anti-apoptotic protein Bcl-2 (56). Conversely, inhibition of miR-21 can chemosensitize human GBM cells to TMZ and other drugs such as paclitaxel, sunitinib, doxorubicin, and VM-26 (57-61).

Three other microRNAs, MiR-195, miR-455-3p, and miR-10a* were described to be involved in TMZ resistance as they were upregulated in a TMZ resistant variant of the U251 glioblastoma cell line (62). Indeed, miR-195 silencing was shown to significantly enhance the TMZ effectiveness.

Similarly, two studies showed that miR-221, miR-222, miR-181b, miR-181c, and miR-128 were significantly downregulated in GBM, while miR-21 was overexpressed (63, 64). MiR-181b and miR-181c had the strongest correlation with responsiveness to TMZ treatment, and therefore could be used as predictive markers for response to TMZ therapy.

In terms of glioma stem cells (GSCs) response to therapy, miR-125b-2 has also been shown to increase resistance of GSCs to TMZ, whereas peptide nucleic acid (PNA) miR-125b inhibitors increase TMZ-induced GSCs apoptosis via mediation of cytochrome c release from the mitochondria, caspase-3, and PARP activation (65).

On the other hand, MiR-100 has been reported to increase the sensitivity of glioma cells to ionizing radiation through the downregulation of ataxia telangiectasia mutated (ATM) (66).

MicroRNAs as therapeutics

It has been demonstrated that both loss and gain of miRNA function contribute to cancer development through the upregulation and silencing, respectively, of different target genes. Experimental evidence indicates that the use of miRNA mimics or anti-microRNAs (anti-miRs or antagomiRs) may represent a powerful therapeutic strategy to interfere with key molecular pathways involved in cancer progression.

Several reports in GBM describe preclinical investigations to characterize individual oncogenic and tumor-suppressive miRNA that can be targeted *in vitro*, with some strong evidence of efficacy in mouse models; however up to date, none of them has advanced to clinical trials in GBM patients.

Most therapeutic strategies targeting oncogenic miRNAs have mainly focused on delivering stabilized antisense oligonucleotides complementary to the miRNAs sequence. Preclinical studies with GBM tumor-suppressive miRNAs have consisted of forced overexpression of miRNA mimics.

MiR-21 and miR-10b figure among the prominent and powerful oncogenic microRNAs targeted in GBMs and described in multiple studies (26, 67-69). Some of these GBM miR-21 and miR-10b studies have demonstrated preclinical efficacy with delivery of miRNA antisense. One particular

study has demonstrated preclinical efficacy in GBM models with a radically different approach to targeting miR-10b; by using viral delivery of CRISPR/Cas9 system to eliminate miR-10b expression in GBM (70).

In terms of microRNAs mimics, more studies have identified tumor-suppressive microRNAs in GBM and shown their potential for therapeutic delivery. Among these molecules, miR-34a has received the most attention in translational studies of tumor-suppressive miRNAs and their therapeutic potential in GBM (23, 29).

Other tumor-suppressive microRNAs tested include miR-326, mir-297, miR-128, and miR-182 (71-75). Most of these microRNAs were investigated in in GBM using *ex vivo* transfection prior to GBM cell implantation in the mouse brain, but some studies have reached the higher bar of demonstrating *in vivo* efficacy with tumor-suppressive miRNA delivery to previously established orthotopic GBM in mice. A number of studies have also shown the potential of miRNA-based therapies to indirectly attack GBM, through its vasculature or via immunotherapeutic effects (76, 77, 78, 53, 54).

The effectiveness of miRNA-based therapies remains perhaps the biggest challenge in GBM management. Some approaches tested preclinically have involved local delivery, sometimes combined with the convection-enhanced delivery (CED) to allow better penetration of the molecules into the tumor and the surrounding tissue. The optimization of these delivery approaches was based on the use of vectors such as adenovirus, lentivirus, or a variety of nanoparticles to transfect GBM cells. These viral or nanoparticle vectors have the advantage to get substantial amount of the payload into GBM cells compared to the delivery of naked mimics or antagomiRs. Interestingly, it should also be highlighted that intravenous delivery of miRNA-based therapeutic vectors might be an alternative for GBM. Indeed, some reports describe approaches targeting the brain vasculature or designed to pass through the blood–brain barrier or locally disrupt it (79, 80).

Although there are numerous preclinical studies on miRNA-based therapeutic strategies for GBM, none has yet moved on to clinical trials in patients. However, miRNA-based therapies have entered clinical trial testing for other cancers, and hopefully GBM should not fall far behind. A miRNA-34a therapeutic entered a Phase I trial, using a liposomal miR-34a mimic, administered twice weekly in 47 patients with advanced solid tumors. The trial achieved a partial response and four cases of stable disease, but it was marked by significant inflammatory side effects requiring immune-suppressive steroid premedication (81). This immune reaction may represent another challenge with miRNA therapeutics in the clinic, and it is hoped that valuable information will be gathered from the analysis of this trial.

In conclusion, this small review provides insights about how micro-RNAs act as oncogenes and tumor suppressor genes and how these findings, along with our increasing understanding of miRNA regulation, can be applied to optimize recent miRNA-based technologies and make them suitable for clinical applications in GBMs and other cancers.

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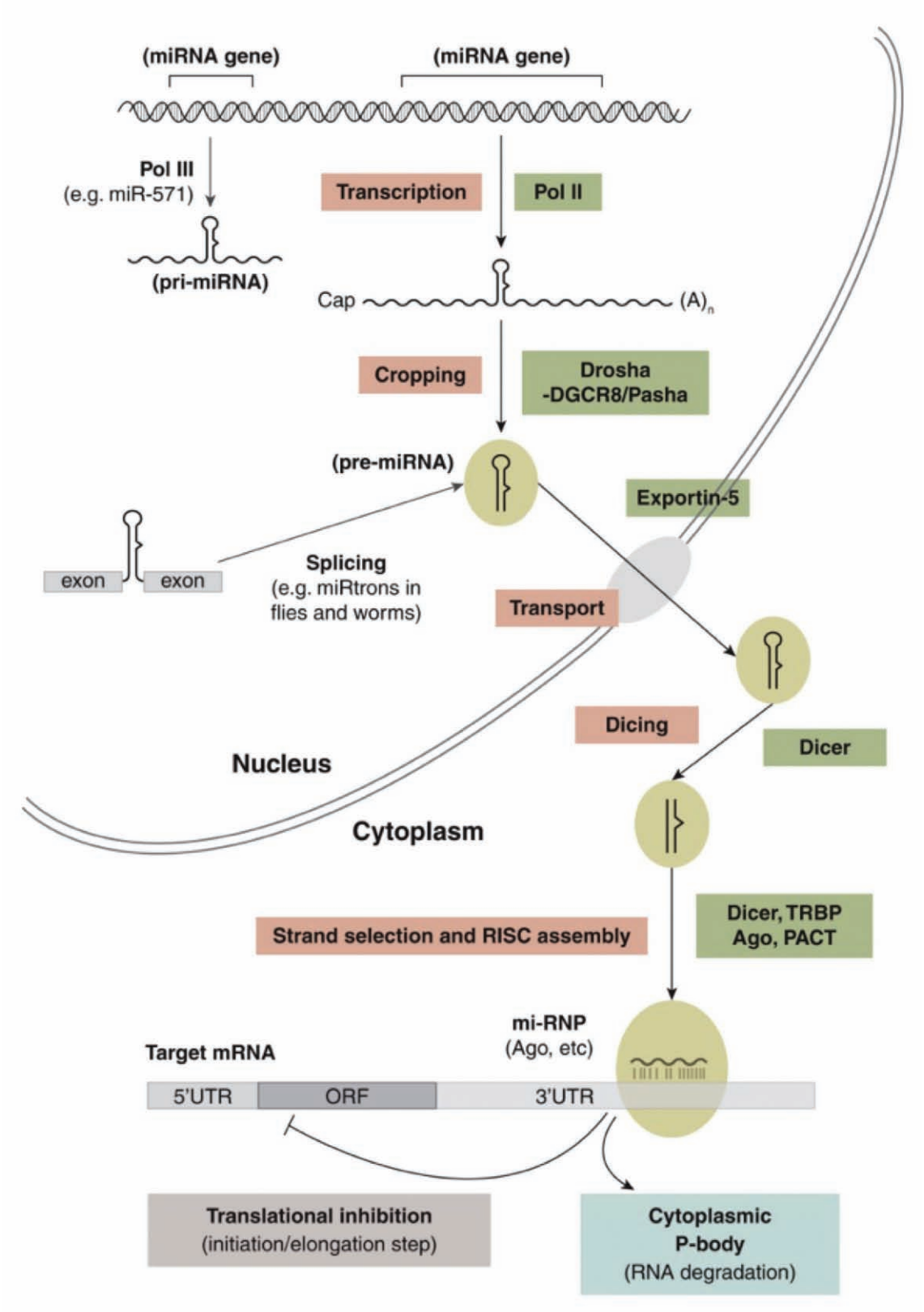
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Dicing
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Nucleus
Cytoplasm
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Target mRNA
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ORF
3'UTR
Translational inhibition
(initiation/elongation step)
Cytoplasmic
P-body
(RNA degradation)

MOLECULAR CHARACTERIZATION OF TRIPLE NEGATIVE BREAST CANCERS: POTENTIAL APPLICATION FOR TARGETED THERAPY

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Definition

The definition of **triple-negative breast cancer (TNBC)** applies to all tumours that lack the expression of ER, PgR (<1% of positive tumour cells, assessed using IHC) or HER2 (assessed by FISH), all of which are molecular targets of therapeutic agents. TNBC account for about 15-20% of all breast cancers (Fig. 1).

TNBC patients show a relatively poorer outcome compared with those with other breast cancer subtypes, since they have an inherently aggressive clinical behaviour and there is lack of recognized molecular targets for therapy. Although they are relatively heterogeneous, chemotherapy is still the primary established treatment option for patients with early-stage or advanced-stage TNBC.

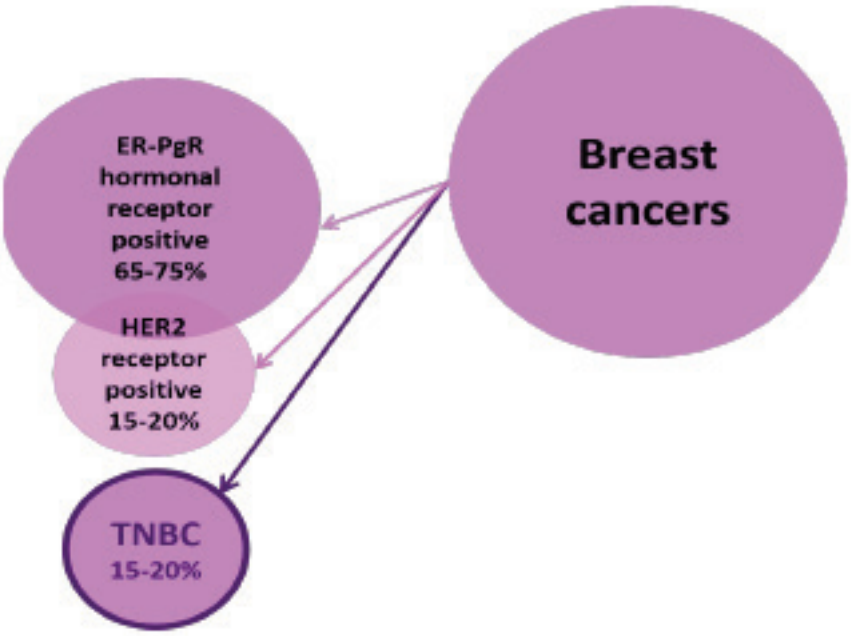

Figure 1. Percentages of breast cancers according to ER-PgR and HER2 receptors positivity

'Basal-like' was the term chosen to define tumours whose cells express genes characteristic of normal basal/myoepithelial cells (KRT5, KRT14 and KRT17; EGFR). More than 90% of **basal-like breast cancers (BLBCs)** are TNBCs and BLBC represents the most frequent subtype of TNBC (55–81%) (Fig. 2). Within TNBC, none of the intrinsic subtypes defined by (Perou et al. Nature 2000; Sorlie et al. PNAS 2001) — including BLBC — differ significantly in terms of the rate of pathological complete response (pCR) or survival after neoadjuvant chemotherapy, and all derive similar benefit from platinum compounds. 50% of BRCA1-2 carriers are basal-like, 90% of TNBC do not have BRCA mutations. Basal-like tumours have distinct molecular features compared with other TNBC subtypes but they also are markedly heterogeneous.

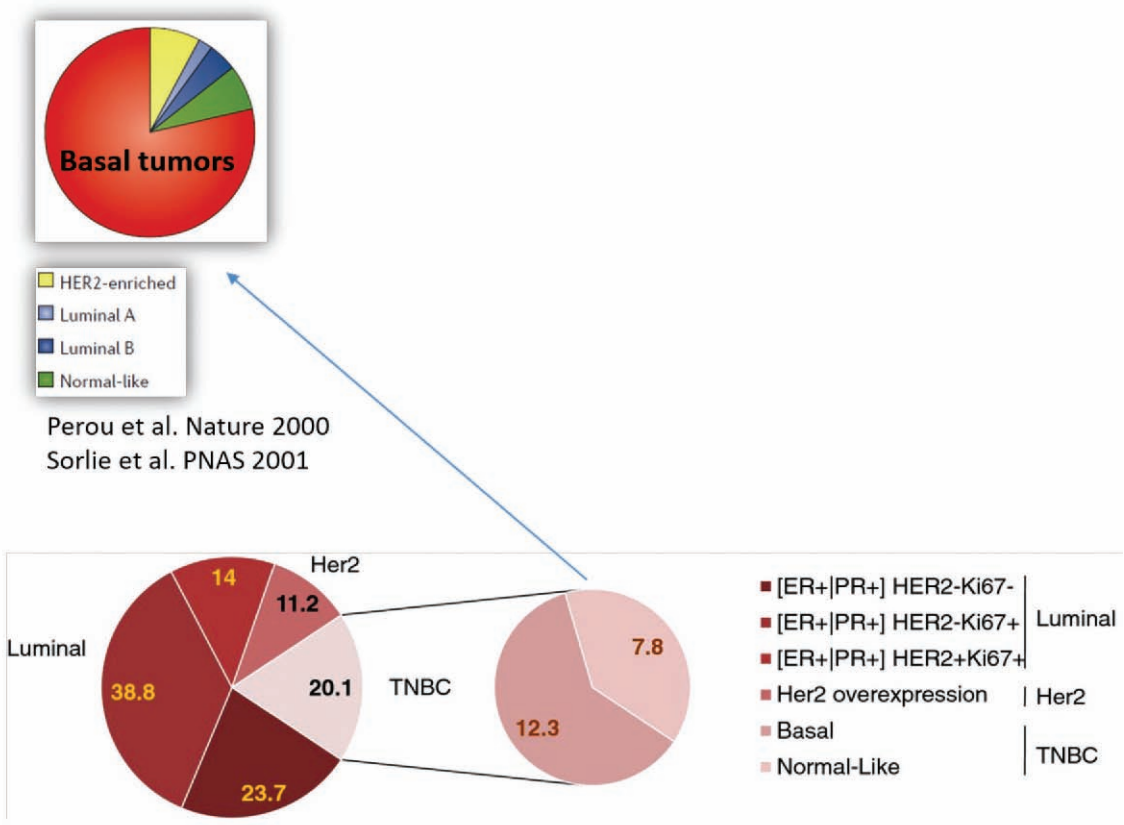

Figure 2. Percentages of luminal, HER2 and TNBC breast cancers.
Basal-like breast cancer represents the most frequent subtype of TNBC

In 2011, Lehman and collaborators proposed a novel classification of TNBC (Lehman et al., J Clin Invest 2011) with 6 subtypes derived from gene expression profiling.

Basal tumors were divided into 2 groups: **basal-like 1 (BL1) and basal-like 2 (BL2)**. The top biological processes for the BL1 subtype are enriched in cell cycle and cell division components and pathways (cell cycle, DNA replication reactome, G2 cell-cycle pathway, RNA polymerase, and G1 to S cell cycle). Elevated DNA damage response (ATR/BRCA) pathways characterize the proliferation pathways in the BL1 subtype. Increased proliferation and cell-cycle checkpoint loss are consistent with the elevated expression of DNA damage response genes. The highly proliferative nature of this subtype is further supported by the finding of high Ki-67 mRNA expression and nuclear Ki-67 staining as assessed by IHC analysis (BL1 + BL2 = 70% vs. other subtypes = 42%; $P < 0.05$). The BL2 subtype displays unique gene ontologies involving growth factor signaling (EGF pathway, NGF pathway, MET pathway, Wnt/β-catenin, and IGF1R pathway) as well as

glycolysis and gluconeogenesis. Likewise, the BL2 subtype is uniquely enriched in growth factor receptor GE such as EGFR, MET, and EPHA2. This subtype has features suggestive of basal/ myoepithelial origin as demonstrated by higher expression levels of TP63 and MME (CD10) (Lehman et al., J Clin Invest 2011).

The **immunomodulatory (IM) subtype** is enriched in immune cell processes, including immune cell signaling (TH1/TH2 pathway, NK cell pathway, B cell receptor [BCR] signaling pathway, DC pathway, and T cell receptor signaling), cytokine signaling (cytokine pathway, IL-12 pathway, and IL-7 pathway), antigen processing and presentation, and signaling through core immune signal transduction pathways (NFKB, TNF, and JAK/STAT signaling). The IM signaling is evidenced by immune signal transduction gene expression, in addition to immune cell-surface antigens, cytokine signaling, complement cascade, chemokine receptors and ligands, and antigen presentation (Lehman et al., J Clin Invest 2011). Since a similar proportion of samples that were microdissected fell into the IM subtype, it is likely that the IM characteristics are unique to the tumor cells themselves and not a reflection of immune cell infiltrate. Immune signaling genes within the IM subtype substantially overlap with a gene signature for **medullary breast cancer**, a rare, histologically distinct form of TNBC that despite its high-grade histology is associated with a favorable prognosis.

The **mesenchymal (M) subtype** displays a variety of unique biological processes that are heavily enriched in components and pathways involved in cell motility (regulation of actin by Rho), ECM receptor interaction, and cell differentiation pathways (Wnt pathway, anaplastic lymphoma kinase [ALK] pathway, and TGF-β signaling). The **mesenchymal stem–like (MSL) subtype** shares enrichment of genes for similar biological processes with the M subtype, including cell motility (Rho pathway), cellular differentiation, and growth pathways (ALK pathway, TGF-β signaling and Wnt/β-catenin pathway). However, unique to the MSL are genes representing components and processes linked to growth factor signaling pathways that include inositol phosphate metabolism, EGFR, PDGF, calcium signaling, G-protein coupled receptor, and ERK1/2 signaling as well as ABC transporter and adipocytokine signalling (Lehman et al., J Clin Invest 2011).

The **luminal androgen receptor (AR) subtype** is ER negative, but gene ontologies are heavily enriched in hormonally regulated pathways including steroid synthesis, porphyrin metabolism, and androgen/estrogen metabolism. AR mRNA was highly expressed, on average at 9-fold greater than all other subtypes. Tumors within the LAR group also expressed numerous downstream AR targets and coactivators (DHCR24, ALCAM, FASN, FKBP5, APOD, PIP, SPDEF, and CLDN8). The percentage of tumor cells scored with nuclear AR staining and the intensity of staining were significantly higher in the LAR subtype (>10-fold; $P < 0.004$) compared with all other TNBC subtypes. Tumors in the LAR subtype display luminal gene expression patterns, with FOXA1, KRT18, and XBP1 among the most highly expressed genes (Lehman et al., J Clin Invest 2011). The LAR TNBC subtype is composed of AR-driven tumors that include the molecular **apocrine subtype**.

TNBCtype is a user- friendly interface created by Vanderbilt University for TNBC subtype prediction, available at http://cbc.mc.vanderbilt.edu/tnbc. Users can classify TNBC tumors or cell line samples by uploading a normalized gene expression data matrix. Input data matrix must consist of gene expression values in a .csv file with gene symbols as rows and sample IDs as columns. After a data format check, the sample is subjected to the ER-filter, to remove any possible ER-positive sample and redo the normalization procedures. Results are displayed as shown in Fig. 3, where each sample is classified as the subtype whose centroid is most correlated with its expression profile.

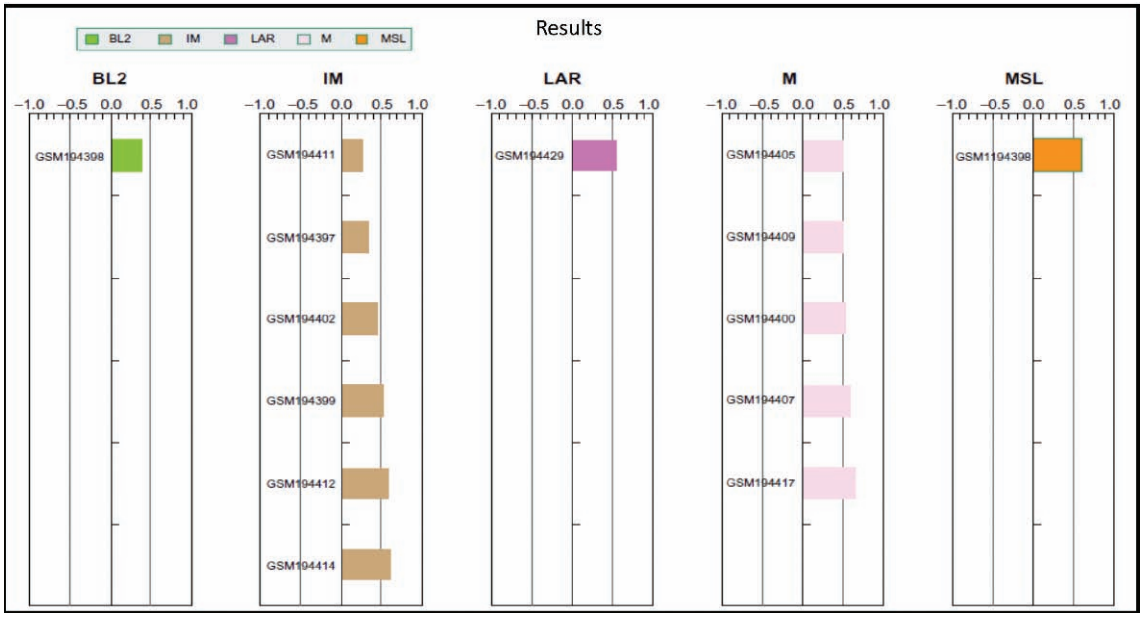

Figure 3. Prediction results for TNBC samples normalized without any ER positive sample. Bars represent Pearson correlation coefficients.

In 2015, Burstein and collaborators proposed another classification (Burstein et al., Clin Cancer Res 2015), where TNCB were subtyped into 4 classes (Fig. 4) according to mRNA expression and DNA profiling: **luminal androgen receptor (LAR)** have CCND1 amplificatrion, **Mesenchymal (MES)** EGFR amplification, **Basal-like immunosuppressed (BLIS)** FGFR2 amplification and **Basal-like immunoactivated (BLIA)** CDK1 amplification.

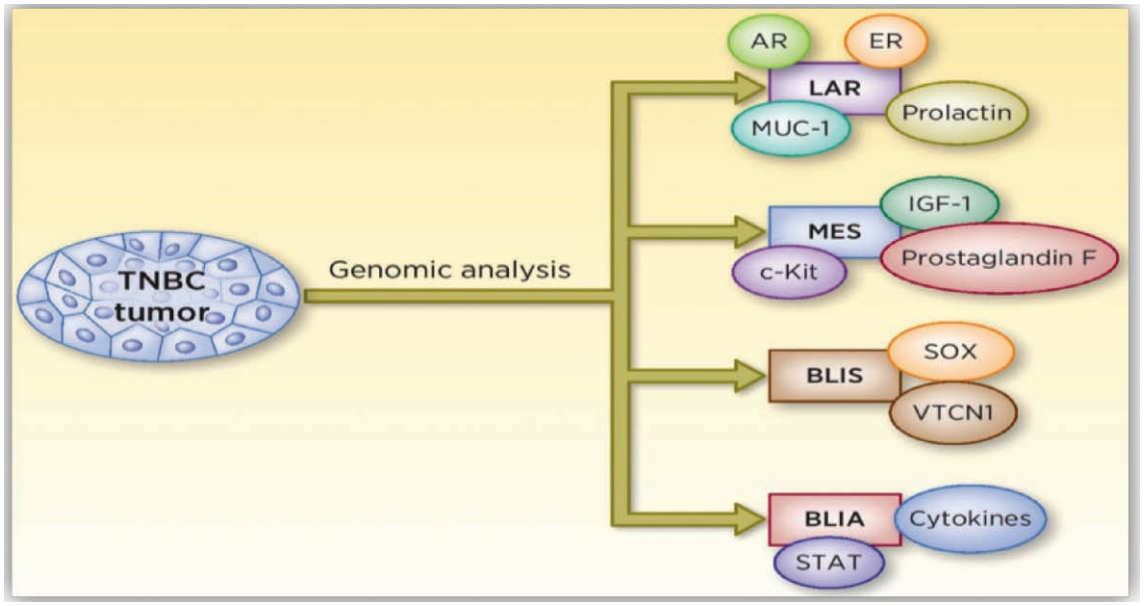

Figure 4. Subtyping of TNBC according to gene expression and DNA profiling (Burstein et al., Clin Cancer Res 2015)

LAR tumors exhibit AR, ER, prolactin, and ErbB4 signaling but lack ERa IHC staining. Gene expression profiling demonstrates expression of ESR1 and other estrogen-regulated genes (PGR, FOXA, XBP1, GATA3). They may respond to traditional antiestrogen therapies as well as to anti-

androgens. MES tumors are characterized by pathways known to be regulated in breast cancer, including cell cycle, mismatch repair, and DNA damage networks, and hereditary breast cancer signaling pathway. In addition, genes normally exclusive to osteocytes (OGN) and adipocytes (ADIPOQ, PLIN1) and important growth factors (IGF1) are highly expressed in this subtype, previously described as "mesenchymal stem–like" or "claudin-low". BLIS tumors exhibit downregulation of B cell, T cell, and NK cell immune-regulating pathways, cytokine pathways and of molecules controlling antigen presentation, immune cell differentiation, and innate and adaptive immune cell communication. However, this cluster uniquely expresses multiple SOX family transcription factors. BLIA tumors display upregulation of genes controlling B cell, T cell, and natural killer cell functions. This subtype has the best prognosis, exhibits activation of STAT transcription factor–mediated pathways, and has high expression of STAT genes.

In 2016, Hon and collaborators defined TNBC that are negative for the androgen receptor as **quadruple negative (QNBC)** (Hon et al. Am J Cancer Res 2016). Unlike AR-positive TNBC, QNBC predominantly exhibit a basal-like molecular subtype. Several subtypes and related pathway proteins are preferentially expressed in QNBC that may serve as effective targets for treatment, such as ACSL4, SKP2 and EGFR. ACSL4 expression has been demonstrated to be inversely correlated with expression of hormone/growth factor receptors and may thus serve as a biomarker for QNBC as well as a target for therapy.

A refinement into 4 tumor-specific subtypes of the initial Lehman's classification was proposed by the same group in 2016 (Lehman et al. PLoS ONE 2016). Histopathological quantification of the stroma component and laser capture microdissection (LCM) indicated that transcripts in the immunomodulatory (IM) subtype derived from infiltrating lymphocytes and transcripts in the mesenchymal stem-like (MSL) subtype from and tumor-associated stromal cells. The **TNBCtype-4** contains BL1, BL2, LAR and M classes only.

In 2018, Bareche and collaborators (Bareche et al., Annals Oncology 2018) used copy-number aberrations, somatic mutations and gene expression data derived from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA) and classified 550 TNBC samples according to Lehmann's molecular subtypes using the TNBCtype tool. Due to its instability, the BL2 subtype was removed, and BL2 samples were reassigned to the second highest significant correlated centroid. BL2 samples were reclassified as LAR (19%), IM (15%), M (11%) and MSL (11%). **BL1 subtype** was found to be most genomically instable subtype with high TP53 mutation (92%) and copy-number deletion in genes involved in DNA repair mechanism (BRCA2, MDM2, PTEN, RB1 and TP53). **IM subtype** was associated with a better prognosis and showed high expression levels of immune signatures and check-point inhibitor genes such as PD1, PDL1 and CTLA4. **LAR subtype** was associated with a worst prognosis, higher mutational burden with significantly enriched mutations in PI3KCA (55%), AKT1 (13%) and CDH1 (13%) genes. **M and MSL subtypes** were associated with higher signature score for angiogenesis.

Among the most frequent mutations in TNBC, only TP53 mutations are found at a high frequency (60–90% of mutations), and are more common in basal-like (62–80%) and in particular in BL1 subtype than in non-basal TNBC (43%). PIK3CA is among the most commonly mutated gene in TNBC (~20%globally), more frequently in LAR TNBCs (55%) than in the other subtypes. Among the mutations occurring at a low frequency in TNBC, some are actionable or are a target of existing therapies. The low frequency of TNBC driven by rare aberrations represents a challenge for ad hoc drug development, and requires the implementation of new trial designs and widespread adoption of genomic screening in clinical practice.

TNBC treatment

TNBC is a very heterogeneous disease, for which a number of therapeutic strategies are being explored (Mayer et al., Clin Cancer Res 2014). TNBC cannot be treated in a uniform fashion; for instance, TNBCs that have a basal-like genotype are more likely sensitive to DNA cross-linking agents, such as platinum-based chemotherapy and PARP inhibitors. On the other hand, the androgen receptor–expressing tumors may derive greater benefit from a combination of an androgen blocker and a PI3K inhibitor. Numerous experimental approaches are under way with the goal of identifying "targets" in TNBC, with PI3K inhibitors, MEK inhibitors, HSP-90 inhibitors, histone deacetylase inhibitors, PD-1 (programmed death 1) inhibitors, etc. under consideration or currently being investigated in the clinical setting. However, TNBC is clearly a complex disease. As such, it is likely that its biology involves multiple redundancies and pathway cross-talk. If only one pathway is selectively inhibited, the efficacy of the therapeutic strategy would likely be undermined by activation of a compensatory pathway. Combining two or more targeted agents may be required for a more rational and optimal approach to TNBC treatment. Efforts in this direction are evidenced by novel clinical trials involving different/complementary pathway inhibitors, such as phase I and II studies combining PI3K inhibitors with PARP inhibitors or with androgen blockers, platinum agents with PI3K or mTOR inhibitors or HDAC inhibitors, etc.

It is generally established that patients with breast cancer who achieve a pCR (lack of residual disease in both breast and axilla) after neoadjuvant therapy exhibit a good long-term outcome. A high residual disease burden (RDB) in the posttreatment, surgically excised cancer has been shown to correlate with a high rate of recurrence and death. More specifically, at least 40% of patients with TNBC who do not achieve a pCR after anthracycline and taxane–based neoadjuvant chemotherapy will have a recurrence within 36 months. However, approximately 30% of TNBC treated with anthracycline and taxane–based chemotherapy will have a pCR after treatment, and consistent with the above data, achieving a pCR to neoadjuvant chemotherapy in this group of patients has been shown to be a strong positive prognostic factor. Patients with TNBC who complete neoadjuvant therapy and have no clinical evidence of metastatic disease after surgical excision of the cancer, regardless of their RDB, are usually observed without further treatment. This conduct might not be appropriate for patients at a very high risk of early recurrence such as those with a high RDB in the residual drug-resistant tumor. However, the appropriate therapy for those patients is unknown, and personalized treatment strategies using adjuvant therapies that molecularly target tumor-specific dependencies are sorely needed. The intertumor heterogeneity of TNBCs before and after neoadjuvant chemotherapy underscores the need for powerful and broad molecular approaches to identify actionable molecular alterations and, in turn, better inform personalized therapy of this aggressive disease. Incorporation of these approaches into clinical studies and eventually standards of care will aid in the prioritization of patients with residual disease after neoadjuvant chemotherapy into rational adjuvant studies. The postneoadjuvant treatment setting could be a valuable source for clinical trials initiated to align patients with treatments best suited to target their cancer subtypes.

TNBC subtyping and therapy

The classification proposed in (Burstein et al., Clin Cancer Res 2015) suggests that AR antagonists and MUC1 vaccines may prove effective for the treatment of AR- and MUC1-overexpressing **LAR tumors**, whereas b-blockers, IGF inhibitors, or PDGFR inhibitors may be useful therapies

for **MES tumors**. Conversely, immune-based strategies (e.g., PD1 or VTCN1 antibodies) may be useful treatments for **BLIS tumors**, whereas STAT inhibitors, cytokine, or cytokine receptor antibodies, or CTLA4 inhibitors may be effective treatments for **BLIA tumors**.

Lehman's subtyping (Lehman et al., J Clin Invest 2011), revised by Bareche and collaborators (Bareche et al., Annals Oncology 2018), suggests that **BL1 tumors** may be sensitive to PARP inhibitors, since they show high genomic instability, high copy number losses for TP53, BRCA1/2 and RB1 genes, as well as high copy number gains for PPAR1 gene. BL1 may also be potential candidates for MEK1/2 inhibitors as 90% of them displayed copy number gains for KRAS, NRAS and BRAF with significant mRNA overexpression of the corresponding genes. Furthermore, they may also benefit from PI3K/AKT inhibitors for the high frequency of PIK3CA copy number gains with significant overexpression of PIK3CA, AKT2 and AKT3 genes, and may be sensitive to CDK1/2 and spliceosome inhibition since they show MYC amplification and overexpression.

LAR and MSL tumours may be potential candidates to CDK4/6 inhibitors since they have RB1, CDK4 and CDK6 expression level alterations. They may benefit from PI3K and AKT inhibitors as previously reported in preclinical models since somatic mutations in PI3K signalling pathway are found in 75% of LAR tumours. LAR subtype may also benefit from CDK1/2 and spliceosome inhibition since they show MYC amplification. MSL tumours may derive benefit from an antiangiogenic therapy in contrast to unselected TNBC population thanks to the overexpression of the "Inducing Angiogenesis" hallmark together with a significant mRNA overexpression of PDGFR and VEGFR that may drive MSL tumorigenesis.

Targeting EGFR and Notch pathways may be an option for **M tumours**, since they show an enrichment of EGFR and Notch signalling pathways, with high level of mRNA expression for EGFR, NOTCH1 and NOTCH3. M and IM may benefit from inhibition of CDK1/2 and of the spliceosome for MYC amplification in M and IM and MYC overexpression in M. **IM tumours** may mostly benefit from checkpoint inhibitors because they have high expression levels of immune related signatures as well as high mRNA expression levels of immune check point inhibitor genes such as PD1, PDL1 and CTLA4.

Immunotherapy

The findings that a population of TNBC is immunogenic and actively engaged by the immune system provides a strong rationale for testing immunotherapies targeting the immune checkpoints to elicit, reinvigorate and potentially expand the magnitude of pre-existing anticancer immune responses. Loss of PTEN expression in TNBCs is associated with PD-L1 overexpression, confirming an association between increased PI3K signalling and the presence of PD-L1. In addition to acquired resistance mechanisms, PD-L1 expression can also be regulated by molecular alterations and oncogenic pathways (intrinsic resistance), linking molecular and immune heterogeneity

Immuno-molecular therapy integrates immune and molecular features to devise novel combinatorial approaches based on targeting intracellular molecular alterations and modulating the immune response. Also chemotherapeutic agents having immunomodulatory activity can act as an immunological adjuvant in the tumour microenvironment to stimulate vaccination-induced antitumour immunity.

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THE MOLECULAR BASIS OF VIRO-INDUCED CARCINOGENESIS

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Abstract

Cancer is the most emerging condition effecting millions of people globally and the leading cause of death. Worldwide, cancer is considered a great public health problem. The etiology of cancer remains multifactorial and implies both the exogenous agents and/or the endogenous factors. A viral etiology of cancer was first demonstrated in the beginning of the 20th century. Overall, epidemiological and clinical studies have shown that eight viruses are linked to human cancer. Collectively, these viruses cause over 20 different types of cancer and contribute to 12-15% of all cancer, with a greater burden in low- and middle-income countries. The main viruses studied are human papillomaviruses associated with cervical cancer, Epstein-Barr virus associated with Burkitt lymphoma and oropharyngeal cancer and Human mammary tumor virus incriminated as an etiological agent of sporadic breast cancer. Although these viruses share similar routes of transmission, they have evolved various mechanisms to ensure efficient viral replication and persistence and evade host immunity. Our increasing understanding of the etiology of virus-mediated carcinogenesis is of a great interest to elaborate prophylactic and therapeutic strategies to reduce the risk of virus-mediated cancer for better management of cancer.

Introduction

Worldwide, cancer is considered as a real health problem and the main cause of death. This disease is caused by the transformation of cells that become abnormal and proliferate excessively leading to the formation of a malignant tumor. Cancer cells can disseminate to anatomically distant organ sites and could subsequently be adapted to foreign tissue microenvironments causing cancer metastasis (1).

Cancerogenesis is a long and multifactorial process. During carcinogenesis, we have a gradual escape of cancer cells from the mechanisms ensuring regulation of cell division and the maintenance of genomic integrity, and neighboring cells also exert a direct or indirect influence

on the process. In cancer clone, cells are immortals, the cell cycle is partially controlled and the differentiation completely altered. The cells no longer obey the contact signals, with disruption of intercellular adhesions and contact inhibition of proliferation. The cancerous cells will also induce neo-angiogenesis and this new vascular network will provide nutrients and oxygen necessary for cell proliferation (2).

At molecular level, cancer is a result of successive mutation events and genetic alterations, disrupting the balance between inhibition and stimulation of cell proliferation. In fact, cell division is regulated by the expression of various genes: oncogenes stimulating and anti-oncogenes inhibiting cell division.

Cancer initiation is a heterogeneous event that could be caused by some physical factors: X rays, UV rays and ionizing radiations; chemical factors: tobacco, alcohol or some chemicals; genetic factors: mutations in some predisposition genes or epigenetic alterations; or viral factors: human papillomavirus, Epstein Barr virus (EBV) or hepatitis B or C viruses (HBV and HCV). Collectively, tumour viruses contribute to 10–12% of all cancers worldwide, among them, 80% are due to HPV and HBV (3).

2. Brief history of oncovirus

Historically, viruses have been defined as simple biological and infectious entities able to cross Chamberland filters with very low porosity. The first virus identified, the tobacco mosaic virus was identified in 1882, but the virus was isolated in 1935. Subsequently, several viruses have been identified affecting humans as well as animals and plants. The first oncogenic virus reported so far was the avian leukemia virus (ALV) which was identified in 1908 by Ellerman and Bang (4). The interest on the etiologic role of viruses in cancer development began in 1970, and the first studies of molecular mechanisms of carcinogenesis were done on Rous sarcoma virus (RSV), responsible of avian sarcoma (5).

Currently, epidemiological and clinical data have identified a number of viruses associated with the development of cancers in humans, namely DNA viruses including EBV, responsible for Burkit's lymphoma and nasopharyngeal cancer; HHV8, responsible for Kaposi's sarcoma; HPV responsible for benign tumors but also several types of cancers (cervix, vagina, anal or penis) and HBV responsible for the development of hepatocarcinoma, and RNA viruses such as HTLV1 responsible for leukemia in adults or HCV involved in the development of hepatocarcinoma.

3. Molecular mechanisms of viro-induced carcinogenesis

During last decades, the molecular mechanisms of viro-induced carcinogenesis study have known a great evolution at both conceptual and methodological levels, using the latest scientific advances and taking advantage of various technological developments. Overall, there are multiple physiological processes that altered by oncoviruses (Figure 1). However, three major molecular mechanisms prevail and are responsible for the malignant transformation of cells: (1) transforming viral proteins; (2) Viral transforming proteins with cellular origin and (3) mutagenesis by insertion. However, viruses could be at the origin of other events that could be involved in tumors' development.

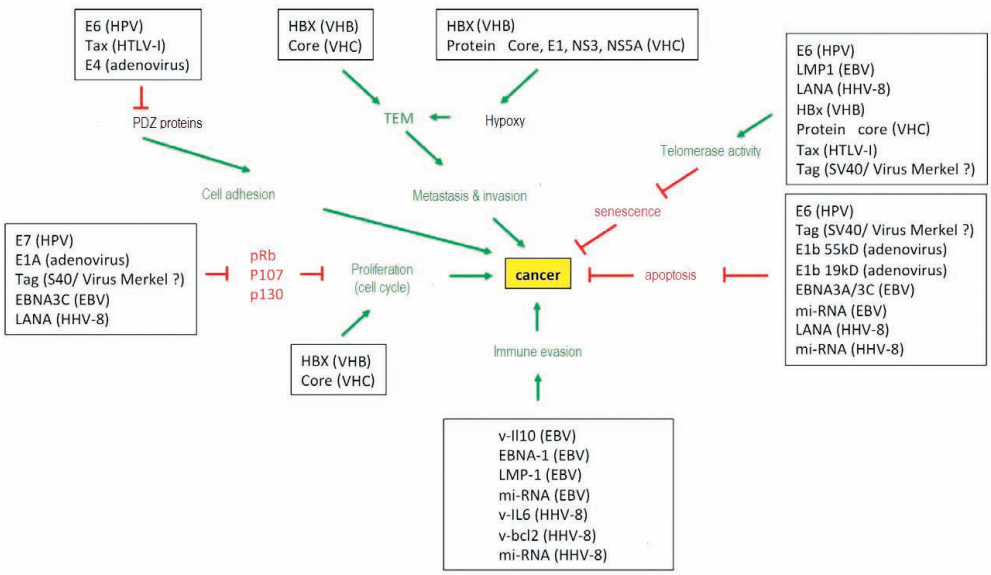

Figure 1. The main signalisation pathways affected by oncoviruses
Adapted from Kalland et al., (6)

3.1. Transforming viral proteins

In some viruses, the genome encodes proteins that directly oncogenic. The mode of action of these oncoproteins is variable and complex: they can interfere in the DNA replication; they can act as transcriptional repressors, some of them are able to activate the expression of some cellular genes; others can have specific biochemical and enzymological properties (e.g. multiple phosphorylations). However, the best known and the most effective mode of action is the ability of these oncoproteins to bind to other cell proteins such as p53 and pRb, which are known to play a crucial role in cell cycle regulation.

The best-studied example is Simian Virus 40 (SV40), widely known as non-oncogenic for humans but able to develop tumors in newborn hamsters. However, this virus is able to transform human cells in vitro. Scientific evidence has shown that the carcinogenesis of SV40 is due to the existence of a protein, called a large T antigen that can bind and therefore inhibit the pRb and p53 host cell proteins (7).

In normal cells, the E2F family regulates the expression of genes involved in the G1/S transition, as well as genes encoding replication-controlling proteins (MCM, cdc6), enzymes necessary for DNA synthesis. In addition, some E2F family proteins (E2F-1, E2F-2 and E2F-3) are potent transcriptional activators that are controlled by pRb. Whereas the other E2F proteins (E2F4 and E2F5) are weaker activators, that are controlled by p107 and p130. In this case, the complex pRb/E2F, inhibits the E2F activity and cell cycle is maintained in G1. In a transformed cell, the phosphorylation of the pRb or its sequestration by a transforming viral protein, releases E2F and allows the progression of the cell cycle through the G1 phase and the G1 / S transition. Two viral proteins with known transformation activity by inactivation of pRb are widely discussed and documented: E7 protein of HPV and T antigen of SV40 (8).

pRb protein could also be activated by the adenovirus e1a protein, which can activate a number of genes required for the entrance into the S phase of cell cycle. Normally, this proliferation is controlled by p53 that arrests the cell cycle and induce cell apoptosis or senescence. However, when the p53 pathway is inactivated, the cells will have transient or continuous proliferation leading to cell immortalisation. Inactivation of p53 may be intrinsic following genetic mutations or epigenetic alterations, or environmental, such as E6 oncoprotein of HPV.

3.2. Viral transforming proteins with cellular origin

The presence of highly similarity between cellular and viral genes leads, after infection, to DNA recombination and genetic rearrangement that could be at the origin of abnormal regulation and initiation of tumours. The most widely studied oncogenic process is the implication of v-sis gene in the PDGF pathway.

The amino acid sequence of the two major polypeptide chains of human PDGF is highly similar to that predicted for the transforming protein encoded by the v-sis oncogene (9). The v-sis product undergoes rapid disulfide-linked dimer formation and further processing of its N and C-termini, yielding a molecule analogous to biologically active PDGF (10). Moreover, the v-sis gene product have been shown to bind PDGF receptors, to stimulate tyrosine phosphorylation of the receptor, and to act as a potent mitogen specific for connective tissue cells that possess PDGF receptors (11, 12).

On the other hand, amino acid sequences of some peptides derived from the RGF receptor were found to exhibit close similarity with portions of the sequence deduced from the v-erb transforming protein (13).

3.3. Mutagenesis by insertion

In some retroviruses, the viral genome usually integrate upstream of the c-myc coding exons and are arranged in the same transcriptional direction, enabling the provirus to utilise its 3' LTR promoter to transcribe and deregulate the downstream c-myc gene. Scientific evidence has shown that c-myc is a proto-oncogene promoting the survival of B lymphocytes, and its overexpression (induced by retroviral insertion) is a predisposing event for the appearance of lymphocytes' tumours. Overexpression of c-myc could also be the results of some DNA translocation as it's the case in Burkitt's lymphoma in particular (14).

4. Epstein-Barr virus.

EBV, also called HHV-4, is a double-stranded DNA gamma-1 herpesvirus. Epstein – Barr virus (EBV) is a human cancer-associated virus that infects > 90% of the global population (15). It is estimated that EBV accounts for more than 200,000 cases of cancer each year and that 1.8% of all cancer deaths are due to EBV associated malignancies (16, 17). Retrospective and prospective epidemiologic studies have indicated an association between EBV and the development of different malignancies and increasing interest has focused on the EBV-associated epithelial cancers that represent 80% of all EBV-associated malignancies, such as Burkitt's lymphoma, 40%–50% of Hodgkin's disease, B-cell lymphoma in immunocompromised individuals, and NPC (18). Among these, nasopharyngeal carcinoma (NPC) is the most common, with 78 000 new cases reported annually worldwide (15).

EBV is transmitted primarily through saliva and is shed intermittently in healthy carriers. Transmissions through breast milk, genital secretions and blood transfusion have also been reported. Primary EBV infection as a result of organ transplantation is a major risk factor for post-transplant lymphoproliferative disease (19).

The EBV genome within LCLs usually exists in multiple copies of extra-chromosomal circular genetic material known as episomes and expresses all latent genes, including six Epstein–Barr nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C and EBNA leader protein (EBNA-LP)), latent membrane proteins LMP1 and LMP2 (which encodes two isoforms, LMP2A and LMP2B) and the non-coding EBV-encoded RNAs (EBER1 and EBER2) and viral microRNA (miRNA) (Figure 2).

EBV remains the most efficient transforming agent; rapidly converting approximately 3–10% of all target B cells into permanently growing lymphoblastoid cell lines (LCLs) (20). During primary infection, EBV initially undergoes a brief replication in the epithelial cells of the oropharynx and salivary glands. The virus subsequently infects trafficking B-cells where the virus establishes a lifelong persistence and proceeds periodic spontaneous reactivation, resulting in lytic replication, infectious virus production and transmission (21, 22).

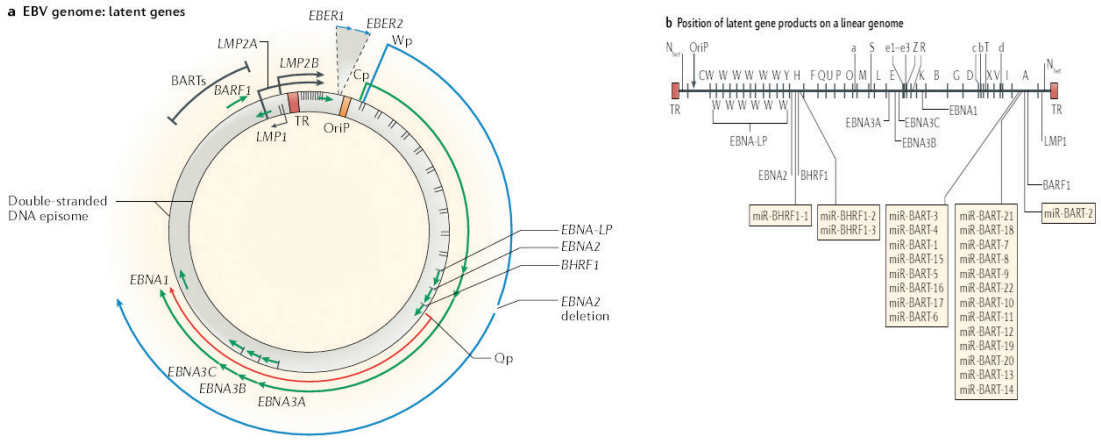

Figure 2. EBV and its latent genes (23)

a. Location and transcription of Epstein–Barr virus (EBV) latent genes on the double-stranded viral DNA episome. B. Location of open reading frames for EBV latent proteins on the BamHI restriction map of the prototype EBV and including the viral microRNAs (miRs) in sequence order.

In latent infection, EBV genomic DNA exists as an episome, replicating only once during S phase and partitioning accurately into daughter cells during the mitotic phase. In lytic state, the EBV genomic DNA is linear. The initiation of lytic replication process greatly depends on the expression of two EBV immediate-early (IE) genes, BZLF1 and BRLF1, whose protein products (Zta and Rta) function as transcriptional transactivators and induce the lytic cascade of viral gene expression (24). Of all the viral transactivators, ZEBRA (also called BZLF1, EB1 and Zta), is unique in initiation of the ordered cascade of EBV genes expression, resulting in the expression of an estimated over 100 viral replication associated genes including those encoding early antigens, viral capsid antigens and membrane antigens. Many target genes of ZEBRA, such as BZLF1, BRLF1 and BMLF1 encoding the transactivators, BHRF1 and BHLF1 encoding the viral homologues of Bcl-2, and BMRF1 encoding EBV DNA polymerase accessory protein (25, 26), have been identified in the EBV genome. Through binding to cisacting AP-1 or ZEBRA responsive elements (ZREs) in lytic cycle promoters ZEBRA activates the transcription of the target genes (27). Recently, some cellular genes modulated by ZEBRA have also been revealed.

The products of these cellular genes are fundamentally linked to the viral life cycle, virus-host interactions, hosT-cell environment, cell cycle progression and immunomodulation. Evidence for a contribution of the lytic cycle to EBV-induced oncogenesis has emerged only in recent decade. Indeed, some studies have reported that the presence of low numbers of infected cells could enhance tumour growth through the release of growth factors and immunosuppressive cytokines. On the other hand, the EBV lytic genes BZLF1, BGLF4 and BGLF5 can also induce genome instability (28).

Recent RNA sequencing and high-throughput PCR array analyses have revealed high-level expression of lytic genes, particularly LF1, LF2, LF3, BILF1, BALF4 and BHLF1, in cell lines and tumour biopsies, although it is not clear whether these genes are expressed in latently infected cells (29).

Currently, a great interest is given to use EBV as a therapeutic target for EBV associated diseases. The development of novel therapeutic approaches using virus reactivation (30), gene therapy (31), drugs that target EBV latent protein function, such as inhibitors of EBNA1, which is known to be expressed in every EBV-positive tumour (32, 33), or therapeutic vaccination (34, 35) provides exciting opportunities for meaningful clinical intervention. The effectiveness of these modalities will be further enhanced by the molecular classification and stratification of cancer patients with EBV-associated tumours.

5. Human papillomavirus

Epidemiological and clinical studies have clearly demonstrated that HPVs are the major etiologic agents of neoplasia of the cutaneous and mucosal epithelia; HPV positivity in cervical cancer is estimated to be more than 95%. Moreover, there is compelling evidence to indicate that the development of human cervical cancer without involvement of the specific HPV is exceptional. (36 - 39). HPVs, particularly HPV 16, are also the cause of multiple cancers in men (penile, anal and a subset of oro-pharyngeal cancers) as well as in women (cervical, vaginal, vulvar, anal and oro-pharyngeal cancers) (40).

HPV is the most common sexually transmitted infection. Worldwide, the prevalence of HPV infection in women with no cervical abnormalities is 11–12% with higher rates in sub-Saharan Africa (24%), Eastern Europe (21%) and Latin America (16%) (41). In Morocco, the overall HPV prevalence was 15.7%, and the most detected genotypes were HPV 16 and 18 (42).

Up to now, more than 200 HPV genotypes were reported, but the interest is focused only on 30 genotypes that are closely associated to cervical lesions' development. Among them, 16 HPV types have been classified as high risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82) and 114 have been classified as low risk genotypes (6, 11, 26, 40, 42, 43, 44, 53, 54, 61, 70, 72, 81, and CP6108) (41). Among the high risk HPV genotypes, HPV 16 is the most common in squamous cell carcinoma of the cervix (50–60%), followed by HPV-18, which is present in about 11–15% of cervical cancer cases (37, 43). Human papillomavirus (HPV) genomes are circular dsDNA characterized by eight open reading frames (ORFs), which are all transcribed from the same DNA strand and orientation, and yield two classes of proteins which are classified as non-structural regulatory proteins (E1-E7) and structural proteins L1 and L2 based on their temporal expression. The intrinsic capacity of L1 proteins to assemble into empty capsid-like structures have been used to develop virus like particles (VLPs) largely used in the induction of protective immunity in animal models and the development of prophylactic vaccines for HPV infection. Accordingly, two prophylactic vaccines; Cervarix (GSK) and Gardasil (Merck); based on the L1 proteins of HPV16 and HPV18, have been introduced into the immunization schedule in many developed and some developing countries (44).

In the pathogenesis of cervical carcinoma, three major factors were identified. Two of them are related to the HPV presence, the effects of viral E6 and E7 proteins, and the consequences of HPV DNA integration in the cellular genome. The third factor is the accumulation of cellular genetic damage, not related to HPV, needed for tumour development (45).

HPV-DNA integration into host chromatin is usually a necessary event in the pathogenesis of HPV-related cervical cancer. It is one of the key stages in malignant progression and is therefore a potential biomarker that precedes invasive disease.

Many studies have demonstrated that the integrated HPV DNA is linearized between the E1 and L1 genes. Upon viral integration, variable parts of the HPV genome are disrupted; fragments containing E2 and E4 ORFs are missing whereas the entire E1, E6 and E7 ORFs are integrated and retained (46).

HPV viral integration is made in such way that the viral regulatory region and the E6 and E7 genes are expressed from viral promoters, but with a different regulation, in which cellular factors might play an important role (45, 46). In the normal HPV life-cycle expression of E5, E6 and E7 is tightly regulated within cells that are destined to be lost from surface epithelial layers, such that they do not pose a carcinogenic threat (46)

The implication of E6 and E7 proteins in cervical cancer progression is mainly due through their interactions with p53, Retinoblastoma protein (pRB) and hTERT. p53 is a transcription factor that regulates cell cycle arrest, apoptosis, senescence, DNA repair and cell metabolism; p53 activity is inhibited by ubiquitin ligase which also ubiquitinates p53 to initiate p53 degradation (Figure 3). In addition to inducing the rapid degradation of p53, E6 also binds to and degrades FADD, preventing the transmission of apoptotic signals via the Fas pathway (47). pRB is a tumour suppressor protein and interacts with transcription factor E2F to repress the transcription of genes required for the S phase of the cell cycle (Figure 1). E7 can also bind to other connected proteins such as p107 and p130 (46). hTERT is a catalytic subunit of the telomerase protein that acts to synthesise telomere ends of linear chromosomes during DNA replication.

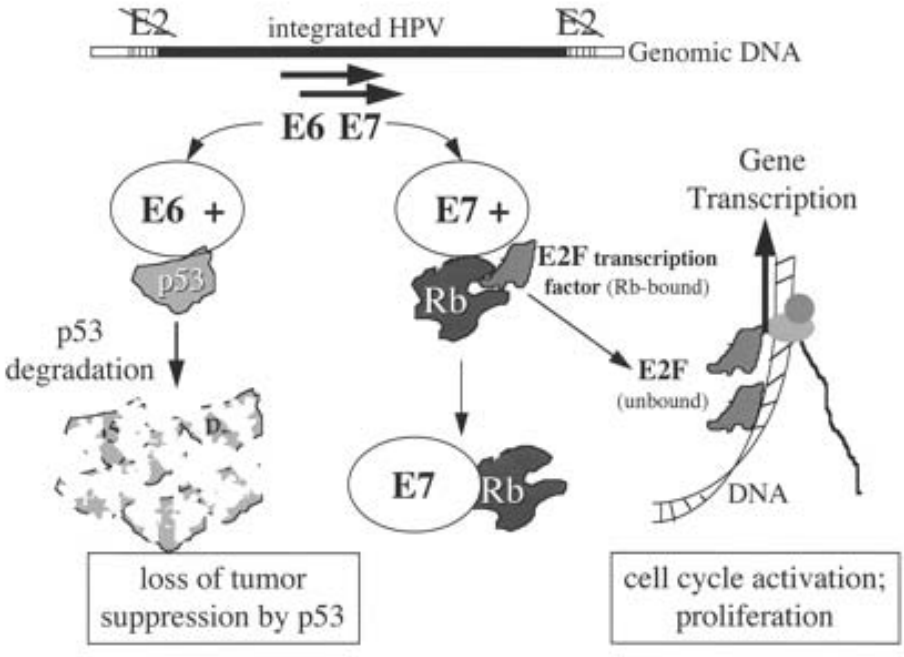

Figure 3. Schematic representation of E6 and E7 activities in cervical cells

Worldwide, cervical lesion diagnosis is mainly based on cyto- and histopathologycal analyses that have largely contributed to decreasing mortality and have been very successful in lowering the incidence rate of cervical cancer in countries with high coverage and good quality control (48). However, conventional cytological and histological diagnosis is even more problematic, because it is difficult to do well, exhibit a low sensitivity and quality sample interpretive errors due to the manipulators subjectivity of the reading of slides, leading to a greater number of interpretive errors (49). Recent achievement in molecular approaches have emerged in clinical practices, are characterized by high sensitivity, specificity and the short time required to perform the procedure, which explain the great interest given to these techniques for HPV testing. These techniques are widely used for HPV detection and/or genotyping, they are based on signal amplification methods (hybridization techniques in liquid phase) or target amplification methods (gene amplification by PCR). The current trend in cervical cancer screening is to improve the sensitivity of screening with new methods and to propose new algorithms for diagnostic and early therapeutic decisions.

6. Hepatitis B and C viruses

Hepatitis B and C viruses infect liver cells and can cause acute and chronic hepatitis B or C, hepatic cirrhosis and hepatocellular carcinoma (HCC).

Chronic HBV or HCV infection can lead to hepatocellular carcinoma (HCC) through a process that induces cell death, regeneration, cirrhosis and, finally, cancer. In many cases, the inflammation induced by chronic infection creates a microenvironment that favors expression of viral oncogenes. For example, in HBV-induced HCC, HBV is clonally integrated into host DNA, and the integrated HBV sequences encode HBV X (HBx) and/or truncated envelope pre-S2/S proteins in a large portion of the HCC. These oncoproteins are thought to participate in directly promoting transformation of hepatocytes to HCC (50).

HBV is an enveloped DNA virus that is a member of the Hepadnaviridae family. Worldwide, approximately 2 billion people are infected with HBV and over 350 million people are chronically infected. Each year, 50 million people will develop new infections (51), and more than one million people die from HBV-related liver cirrhosis and liver cancer (52). Chronic hepatitis B infection develops in individuals who are not able to clear the virus. Overall, in 85%–90% of HBV-related HCCs the HBV genome is integrated into the cellular genome. However, the integrated form of HBV is also present in non-tumor tissue of patients with chronic HBV infections. Integration of the HBV genome into hepatocytes occurs during persistent HBV infection and precedes development of HCC (53, 54). HBV integration leads to the elevated expression of several cellular cancer-related genes, such as TERT, mixed-lineage leukemia 4 (MLL4) and CCNE1 (encoding cyclin E1). HBV integration is also associated with early onset of HCC and poor outcomes, and integrated HBV sequences encoding the HBx and/or truncated envelope pre-S2/S proteins are found in a large percentage of HCC (55).

Integration of HBV into the human genome can occur near or within fragile sites in genes that regulate cell signaling, proliferation and viability. Common targets of integration include genes for human cyclin A2, the PDGF receptor, calcium signaling-related genes, mixed lineage leukemia encoding genes, 60S ribosomal protein genes, human telomerase reverse transcriptase (hTERT) and the retinoic acid receptor β (56).

HCV, a single-stranded enveloped RNA virus that belongs to the Flaviviridae family, was identified in the late 1980s/early 1990s as another virus that causes hepatitis (non-A, non-B) (57). In the absence of treatment, approximately 55–85% people infected with HCV are not able to clear

the virus within six months and develop chronic infection. Among people with chronic hepatitis C infection, 20–30% will develop liver cirrhosis, 1% of these people will develop HCC (32). Viral carcinogenesis is mainly due to the activity of the NS5A viral oncoprotein that is involved in apoptosis inhibition, signal transduction, transcription, transformation and the production of reactive oxygen species (ROS). In particular, NS5A has been shown to bind directly to p53 and to repress transcription of the tumor suppressor p21WAF1 in a p53-dependent manner (50, 58, 59).

7. Human Mammary Tumor Virus

MMTV, an enveloped RNA virus that belongs to the β-retroviridae family, is strongly associated with the development of mouse mammary tumors in both wild and inbred mice. MMTV may be transmitted endogenously through the germline or exogenously through the mother's milk to newborn pups; MMTV virions (intact fully formed replicable but nonintegrated viruses) are ingested into the gut and enter the lymphatic system through lymphocytes and dendritic cells in the Peyer's patches; MMTV-infected lymphocytes move to the spleen where they remain dormant for long periods then possibly during mouse puberty; and the infected lymphocytes move to the mammary glands where the MMTV integrates into the DNA of the host mammary epithelial cells (60). Although the integration of MMTV proviral DNA is thought to be essentially random, integration of an MMTV provirus in the vicinity of a number of host oncogenes, particularly near the Wnt and Fgf family genes, results in inappropriate oncogene expression and clonal outgrowth of the infected cell. Moreover, it has been shown that in addition to activation of cellular proto-oncogenes such as Wnt-1, MMTV can contribute to mammary tumorigenesis by direct transformation of normal human epithelial cells by expression of signaling proteins (61). Moreover, evidence has been presented that the MMTV env protein participates in mammary epithelial cell transformation in vivo using a transgenic mouse model (62). Thus, there may be more than one mechanism by which MMTV causes mammary tumors in mice.

By 2000s, the interest on the viral etiology of human breast cancer has gained a growing interest with the discovery of DNA sequences showing homology to those of MMTV virus in human breast cancer, suggesting that this virus called MMTV-like, also called Human Mammary Tumor Virus (HMTV), could be the human form of the MMTV and may be involved in the development of human breast cancer (63, 64).

Several studies have demonstrated the presence of MMTV-like env sequences in 30– 40% of breast cancer cases in several Western countries including the United States, Italy, Brazil and Argentina (65). Overall, the prevalence of MMTV-like ranges between 78% in Australia to 0% in Iran, Mexico, Germany and Japan (66 - 70).

In African countries, data on the epidemiology of MMTV-like is still limited. In Tunisia, 14% of breast cancer cases were MMTV-like positives (71). In Morocco, MMTV-like sequences were detected in 57.14% of breast cancer cases (72) and in 40% of breast milk samples (not published data).

Of particular interest, prevalence of MMTV-like is reported to be related to the prevalence of *Mus domesticus* in these regions. In fact, it's widely accepted that mice might act as a reservoir and transmit the virus to humans. Moreover, human breast cancer is higher in geographic areas (e.g., Western Europe, USA) where *Mus domesticus* is the most prevalent mouse species than other regions (e.g., Asia) and that Mus domesticus mice produce more MMTV as they carry more exogenous virus and have more endogenous proviral loci than *Mus musculus* (73).

Conclusion

Worldwide, viruses are the causative agents of about 15% of human cancers. Oncogene viruses have developed many strategies for cell immortalization. Better understanding of viral cycle, carcinogenesis mechanism, infection – cancer transition and cell reprograming process are of a great interest to elaborate prophylactic and therapeutic strategies to reduce the risk of virus-mediated cancer for better management of cancer.

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ASSESSMENT OF MOLECULAR BIOMARKERS FOR BLADDER CANCER DIAGNOSIS, GRADING AND PROGNOSIS

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Abstract

Worldwide, bladder cancer is a very significant public health problem, in terms of prevalence, mortality and management for individuals and their families. Bladder cancer is the fourth most commonly diagnosed cancer in men and one of the heaviest cancers in terms of cost. Although transurethral resection of the bladder followed by intravesical instillation of live attenuated Bacillus Calmette–Guérin (BCG) is considered as the gold standard for patients with intermediate and high risk, only a small portion of patient responds to the BCG-therapy. Bladder cancer management is faced by the therapy failure, great side effects and some difficulties in histological classification. Accordingly, growing interest is given to the use of genetic, epigenetic and immunologic biomarkers for molecular signature characterization and tumor stratification. In Morocco, great efforts have been made to contribute to the improvement of management of bladder cancer and many studies were made to evaluate some genetic and epigenetic biomarkers. Generated data is of a great interest for characterization of bladder cancer tumors in Morocco and could be used for better management of this disease.

Keywords: bladder cancer, biomarker, Morocco, tumor stratification, molecular signature.

Bladder cancer

Bladder cancer is the fourth most commonly diagnosed cancer in men and the ninth most common cancer in the world accounting for approximately 420,000 new cases each year (1). Urothelial carcinoma (UC) is so the most frequent histological type of bladder cancer with more than 90% (70% in women and 82% in men) and about 5% of squamous cell carcinoma (SCC) (2). Although rates of bladder cancer are higher in white populations than in other ethnicities, survival is worse for black individuals (3).

In Morocco, bladder cancer is a very significant public health problem, in terms of prevalence, mortality, impact on quality of life for individuals and their families, and economic cost (4).

According to the latest WHO data published in 2017 Bladder Cancer Deaths in Morocco reached 984 or 0.56% of total deaths. The age adjusted Death Rate is 3.47 per 100,000 of population, ranking Morocco the 46th in the world (5). In reference to the regional cancer registers, bladder cancer is the most common cancer with an incidence of 5.8 and 11.3 per 100,000 persons in Casablanca and Rabat, respectively, and the most frequent histological type is by far transitional cell carcinoma diagnosed usually at stages I and II (6-7). The occurrence of bladder cancer increases steadily with age from 45 years with an average age of 65 years. The age-standardized incidence rate (ASR) was 9.7 per 100,000 persons in men and 0.7 in women throughout the years 2006-2008 (4). A study held for eleven years period by Hami et al. between 1994 and 2004 at Al azhar Oncology Center in Rabat on 235 cases states that 82.1% were male and that the age of diagnosis 62.7±12.7 years, they also concluded that bladder cancer in Morocco was the fourth cause of death (8).

Risk factors associated with the development of bladder cancer include carcinogens in tobacco smoke which accounts for about 50% of cases in developed countries (9), and to a lesser extent exposure to chemical compounds in the chemical and rubber industries (10).

Bladder cancer diagnosis

Most bladder cancers are diagnosed after patients present with visible blood in the urine (macroscopic hematuria) or blood found on urine testing (microscopic hematuria) which occurs in 13.7% and 78.3% of patients, respectively (11), and cases are confirmed after transurethral resection of bladder tumor (TURBT), which also serves as the first stage of treatment (12). Several techniques exist to diagnose bladder cancer. As a noninvasive test, urine cytology evaluation allows to confirm whether cells that are shed can be observed for any abnormalities or malignancies (13). As invasive tests, cystoscopy followed by biopsy is the gold standard for diagnosis of bladder cancer (13). Currently, two forms of cystoscopy are available: white light cystoscopy and fluorescence cystoscopy. While papillary tumors can almost always be seen using white light cystoscopy, other forms of tumor require fluorescence cystoscopy to be seen (14). Other imaging modalities used for the diagnosis of bladder cancers are computed tomography (CT), magnetic resonance imaging, and ultrasound. A combination of CT with cystoscopy improves diagnosis of bladder cancer to 100% with 94% specificity (15). The obtained biopsy sample during cystoscopy is histologically evaluated for confirmation, grading, and staging of bladder tumor. The stage classification differentiates between non-muscle invasive (NMI; Tis, Ta, and T1) and muscle-invasive tumors (T2, T3, and T4) according to the invasion depth. Ta tumors are restricted to the urothelium; T1 tumors have invaded the lamina propria; and T2, T3, and T4 tumors have invaded the superficial muscle, perivesical fat, and surrounding organs, respectively. Tis is poorly understood and believed to be a precursor of muscle-invasive tumors. The majority of patients, 70%, initially present with NMI tumors, however, up to 70% of these develop local recurrences, and patients may have several recurrences (16).

Molecular pathways of bladder cancers

Bladder cancer development has traditionally been thought to progress along two distinct genetic pathways that pose distinct challenges for clinical management characterize the evolution of early stage bladder neoplasms (17), one leading to superficial papillary cancers and the other to muscle-invasive cancers.

Superficial bladder cancers demonstrate mainly papillary histology with FGFR3 somatic alterations (up to 80% of stage Ta tumours), wild-type TP53 (inactivating mutations present in less than 15%), Ras pathway activation and stable genomes. FGFR3/HRAS mutation frequently occurs during the development of hyperplasia. A small proportion of these tumours (10–15%) evolve into high-grade muscle-invasive cancers. In case of low-grade Ta carcinoma with recurrent PIK3CA/STAG2 mutation, hyperplasia develops into high-grade Ta carcinoma, which may progress to T1 carcinoma. CIS is characterized by the loss of tumour suppressor genes TP53 and RB1. Further acquisition of additional molecular abnormalities leads to muscle invasive bladder cancers, which are characterized by additional mutations and copy number instability sach as deletions or mutations of the TP53, RB1, ERBB2, or PTEN (18-19) (Figure 1).

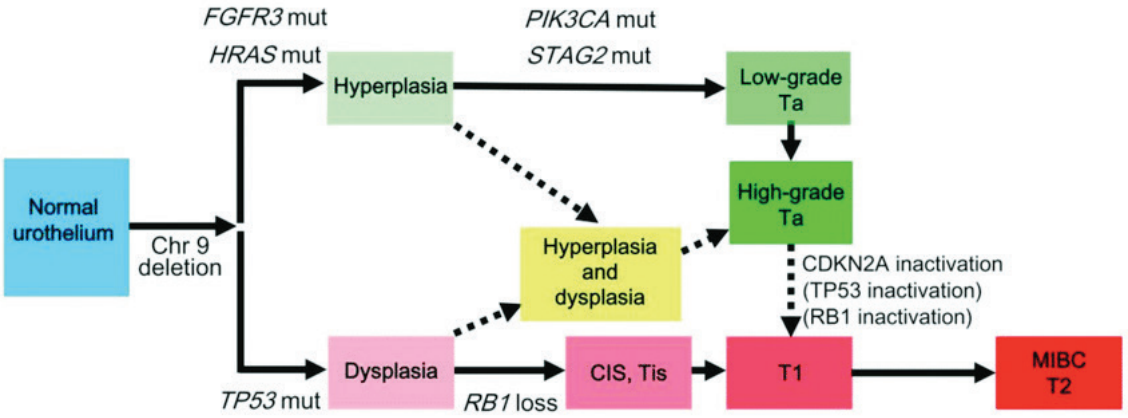

Figure 1: Potential pathways of the tumorigenesis and tumor progression of bladder cancer (19)

Bladder cancer classification: histological vs molecular

Until 2016, different bladder tumor grading systems have been introduced, with generally good acceptance. One of the most widely accepted systems has been the WHO 1973 system, which is still used in some parts of Europe (20). However, the currently recommended system is the new WHO 2016 Grading system, which is being proposed for universal use and should be adopted world-wide (21). Urothelial cell carcinoma patients are stratified by pathologic stage and grade; the basis of clinical decision-making. A large study carried in 1983 showed that 50% of pathologists graded differently the same tumors and that the same tumor was graded differently at different times by the same pathologist, stating that inter- and intraindividual inconsistency in the grading of bladder tumors might invalidate the usefulness of histologic grading in clinical decision making (22). To improve the accuracy of grading the WHO adopted a new classification system for grade in 2004 (papillary urothelial neoplasm of low malignant potential, low grade, and high grade, urothelial cancer) (23). Although, predicting recurrence, progression or response to treatment, continue to pose a real challenge to pathologists (Figure 2).

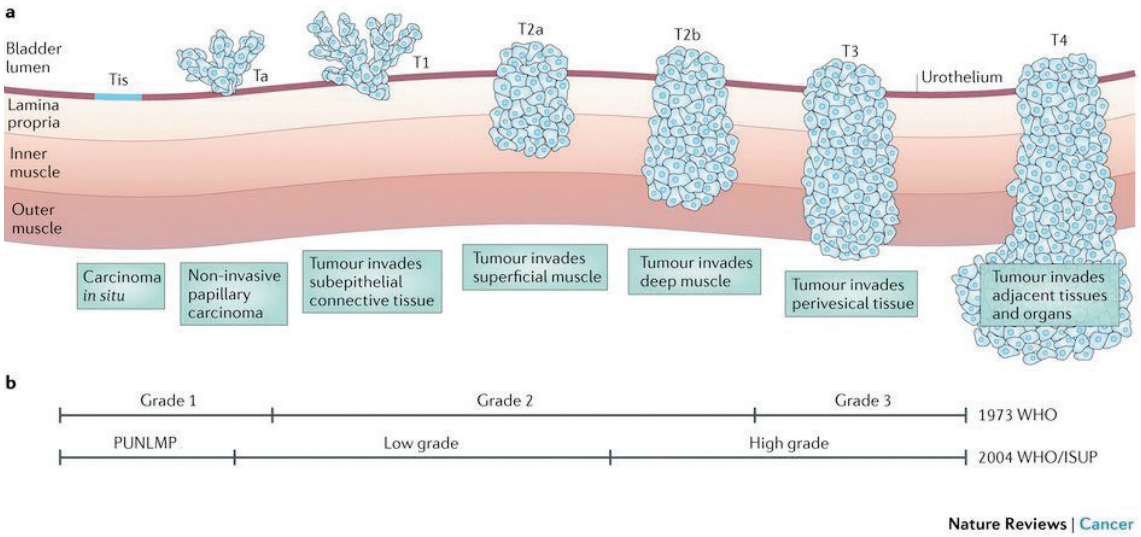

Figure 2: Bladder cancer WHO 1973 and 2004 staging of urothelial tumors (24)
a) Staging of bladder cancer according to the Tumour-Node-Metastasis (TNM) system
b) Grading according to the 1973 World Health Organization (WHO) and 2004 WHO/ International Society of Urological Pathology (ISUP).

The major difference is in the classification of papillary tumours, which are classified as grades 1, 2 and 3 in the older system and as papillary urothelial malignancy of low malignant potential (PUNLMP; equivalent to grade 1), low-grade papillary urothelial carcinoma or high-grade papillary urothelial carcinoma in the WHO/ISUP 2004 classification (24).

Since bladder cancers are biologically heterogeneous and have widely variable patterns, many groups have directed their research into finding a novel classification based on molecular signature that will permit better understanding and management of the disease. Recently, a molecular taxonomy for urothelial carcinoma based on integrated genomics using gene expression profiles could define five major urothelial carcinoma subtypes: urobasal A, genomically unstable, urobasal B, squamous cell carcinoma like, and an infiltrated class of tumors. The subtypes show distinct clinical outcomes suggesting that the molecular phenotypes can be an intrinsic property of the tumors (25). Also, in 2013 Damrauer et al., have demonstrated that there are at least two molecularly and clinically distinct subtypes of high-grade bladder cancer termed "luminal" and "basal-like", which have characteristics of different stages of urothelial differentiation, reflecting the luminal and basal-like molecular subtypes of breast cancer, with clinically meaningful differences in outcome (26). The whole genome mRNA expression profiling and unsupervised hierarchical cluster analyses carried on three data cohorts (Chungbuk, Lund and UCSF) and a discovery cohort of 73 tumors discovered three molecular subtypes of MIB that resembles established molecular subtypes of breast cancer: luminal, basal and P53-like MIBC which was consistently resistant to neoadjuvant chemotherapy (27). In 2017 Sjodahl et al., analyzed 307 advanced bladder cancers both by genome gene expression analysis and by immunohistochemistry with antibodies for 28 proteins and describe five tumor cell phenotypes of advanced urothelial carcinoma: urothelial-like, genomically unstable, basal/SCC like, mesenchymal like, and small cell/neuroendocrine-like, and showed that cancers with different tumor cell phenotypes may converge to the global mRNA analyses, while cases with identical tumor cell phenotypes may diverge (28). Recently, a Meta-cohort Analysis of 2411 unique tumors encompassing NMIBC and MIBC tumors was analyzed, based on gene expression. The analysis revealed six molecular subtypes with different overall survival and molecular features. They have also shown that NMIBC, with a high risk of

progression, displays the molecular features of MIBC (29). Several other groups also sought to define molecular subtypes (Table 1) (30).

UNC [15]	Lund et al. [16]	MD Anderson [17]	TCGA [14]	Broad Institute [18]	Genomic characteristics
Basal	Squamous-cell like	basal	cluster 3	basal	RB1mutations. NFE2L2mutations. p16 deletions. Activating FGFR3mutations. High PD-L1 expression. CDKN2A deletion
	Urobasal B		cluster 4		
Luminal	Unfiltrated	p53-like	cluster 2	immune undifferentiated Luminal immune	
	Genomically unstable	luminal	cluster 1	luminal	P53, RB1 deletions. PPARG, GATA3, ERBB2, E2F3/SOX4 amplifications. ERCC2 alterations.
	Urobasal A				PIK3CA, NFE2L2, ERBB2, ERBB3 mutations. Activating FGFR3 mutations. KDM6A alterations

Table 1: Urothelial carcinoma molecular subtyping classifications (30)

The previously mentioned studies have based their finding on genetic, epigenetic, transcriptomic and proteomic studies. Differences in results in established subtypes is due to differences in study targets, methods and interpretation. All studies confirm the need of biomarkers that can be used to orientate patient treatment and avoid unnecessary toxicities in those who resist. It is also primordial to consider the molecular classification in parallel to histology in decision making.

Progress in Morocco

In the last two decades, many groups in Morocco have focused on the study of bladder cancer in view to contribute to the improvement of standard of care of Moroccan patient. The WHO reported that Schistosomiasis has been eliminated in Morocco, as no new cases were reported since 2004 (31). A study carried in the region of Fez-Boulmane revealed that bladder cancer was the leading cancer with a percentage of 9% of 5532 cases of cancer collected from 2004 to 2010 (32). Casablanca's register showed that bladder cancer is the third most common cancer in men in region between 2005 and 2007 with an age-standardized rate (ASR) of 8.7/100000 whereas the first published population-based data study spanning two years (2006-2008) in the region of Rabat ranked bladder cancer as the fourth malignancy in men with an ASR of 9.7/100000 (6, 33).

Many researchers have explored the genetic and epigenetic properties of bladder patients. In Rabat, El ochi et al., have evaluated HER2 protein expression in 103 bladder cancer cases by immunohistochemistry using HER2 antibody and found that the protein was overexpressed in in 11.7% of cases and was associated with high grade tumors (34).

During the last decade, CNESTEN has given a special interest on bladder cancer research and has identified this topic as a research priority. Accordingly, we have set-up a global strategy to evaluate some biomarkers to be used in cancer diagnosis, molecular staging, cancer prognosis or therapeutic targets.

In this field, the characterization of DNA mutations, SNP and epigenetic events in several genes known for their involvement in tumor progression was performed in bladder cancer specimens and/or exfoliated urine sediments as matrix for non-invasive molecular research.

In this field, the mutational status of *EGFR* exons 18-21 was assessed and has revealed the presence of G2607A polymorphism in exon 20 that was significantly different in patients and healthy controls (35).

The analysis of the mutational status of *FGFR3* and *HRAS* genes in tumor tissues by PCR and direct sequencing revealed that 28.6% and 9.5% of patients carried mutations in *FGFR3* (exon 7 and/or 10) and *HRAS* (exon 1 and/or 2), respectively. To assess the value of the individual markers for non-invasive detection of bladder cancer, DNA isolated from paired urine sediments were also analyzed and have reported a sensitivity of 56.25% for both *FGFR3* and *HRAS* genes.

Moreover, FGFR3 mutations were predominant in both low stage and low grade, whereas HRAS mutations prevail in high grade, suggesting that FGFR3 mutation may be considered as an early biomarker for bladder cancer and HRAS mutation may inform on the prognosis of this cancer (36).

p53 codon 72 and GPX1 Pro189Leu polymorphisms were also evaluated and showed the absence of significant association between patients and healthy controls groups, suggesting that these polymorphisms are not involved in the occurrence of bladder cancer (37, 38).

Evaluation of epigenetic alterations was limited to the methylation status of the promoter region of some genes, including *APC*, *RARB*, Survivin and *GSTP* genes.

For *APC*, *RARB* and *Survivin* genes, methylation specific PCR (MSP) was conducted on both bladder cancer biopsies and paired exfoliated urine cells. Methylation frequencies of the tested genes in tumor specimens were 100%, 75% and 84.4% for APC, RARB; and *Survivin*, respectively and 79.3% 70.8% and 96.3% in urine sediments, revealing a sensitivity of 93.7% of urine sediments as a matrix for molecular research (39).

GSTP1, as a redox biomarker, was also evaluated. The GSTP1 expression was assessed by immunohistochemistry and showed different levels of expression in bladder cancer cases. However, MSP analysis of the promoter region of GSTP1 gene showed the absence of methylation events in all studied cases, suggesting that difference in GSTP1 expression among bladder cancer cases is not due to GSTP1 promoter methylation (40)

Finally, an interest was also given to the viral infection in bladder cancer. Overall, 52.4% of bladder cancer patients were positive for HPV with no significant correlation between the viral infection and tumor stage or grade. Of particular interest, subsequent DNA sequencing of positive cases of HPV revealed the presence of HPV16 in 95.5% of bladder tumor samples (41)

Conclusion

Overall, molecular characterization of biomarkers highlights their potential use in the management of bladder cancer. These biomarkers present an interesting high value for early diagnosis, prognosis, molecular staging and as a potential therapeutic targets. In Morocco, as it's the case in many other developing countries, more efforts have to be made to reinforce the use of molecular biomarkers and to integrate molecular investigation in the global management of bladder cancer in the perspective to implement personalized treatment based on individual molecular signature. Moreover, there's an interesting need to raise research on bladder cancer in Morocco to include immunogenetic aspects and molecular pathways regulation.

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EVALUATION OF GPX1 PRO198LEU POLYMORPHISM, GSTP1 EXPRESSION AND GENE PROMOTER METHYLATION IN MOROCCAN PATIENTS WITH BLADDER CANCER

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Running head: Evaluation of the implication of GPX1 and GSTP1 biomarkers in bladder cancer in Morocco.

Abstract

Bladder cancer (BC) is the third most common male malignancy in Morocco. The risk factors for developing BC are multiples including dietary conditions, environmental exposure and oxidative stress. Glutathione Peroxidase-1 (GPX1) and Glutathione S-Transferase Pi (GSTP1) are two key enzymes in cell detoxification process. GPX1 Pro198Leu polymorphism is associated with a decrease of enzyme activity and may contribute to BC susceptibility. Deregulated expression of GSTP1 enzyme was reported in various human tumors, also, epigenetic silencing of GSTP1 gene by aberrant promoter methylation has been shown to be involved in the molecular pathway for cancer development. In this study, we aimed to assess the presence of GPX1 Pro198Leu polymorphism and determine the expression status of GSTP1-in relation to its promoter methylation- in Moroccan population to evaluate their association with the risk of developing BC in Moroccan patients. Genotyping of GPX1 Pro198Leu polymorphism was carried out by Sanger sequencing. GSTP1 expression was assessed by immunohistochemistry, GSTP1 promoter methylation status was studied by Methylation Specifiq PCR method. No significant association between GPX1 Pro198Leu polymorphism and BC occurrence was found (Pro/Leu vs. Pro/Pro: p=0.425). For the analysis of Pro198Leu polymorphism and progression of BC, no association was observed neither for stages (Pro/Leu vs. Pro/Pro: p=0.500) nor grades (Pro/Leu vs. Pro/Pro: p=0.415). GSTP1 expression was strong in 23.33%, moderate in 60% and weak in 13.33% of BC cases. Variability of the expression does not correlate with high-grade cancer or invasive-stage (p>0.05). No GSTP1 promoter methylation was detected in all cases. Our results showed that GPX1 Pro198Leu polymorphism and GSTP1 expression are not closely associated with the risk of BC in our population, suggesting that the effect of these biomarkers on BC development might be a result of a combination with other genetic and epigenetic alterations and/or non-genetic variables such as diet and lifestyle factors.

Key words: bladder cancer, oxidative stress, gpx1 pro198leu, gstp1 expression, morocco.

Introduction

Bladder cancer (BC) ranks fourth in worldwide cancer incidence for men, being even so very rare in women (1). In Morocco, BC is one of the most frequently diagnosed male malignancies. The commonest histological type is urothelial carcinoma, which occurs with either superficial or invasive phenotypes (2, 3). Etiologically, tobacco smoking, occupational exposure to specific industrial chemicals, chronic urinary tract infections, pelvic radiation and diet intake are known to promote bladder cancer carcinogenesis (4, 5); these potential risk factors are supposed to accumulate, directly or indirectly, reactive oxygen species (ROS) in cells, leading to cellular oxidative stress (6-8). Human cellular Glutathione Peroxidase-1 (GPX1) and Glutathione S-Transferase Pi (GSTP1) are two cytosolic detoxifying enzymes that play a critical role in maintaining cell integrity and protecting DNA from genotoxic and cell-damaging molecules, they inactivate a wide variety of electrophilic carcinogens or stress- induced toxic intermediates by catalysing their conjugation with reduced glutathione and making them easy for secretion (8).

GPX1 gene is located on chromosome 3p21.3 and contains two exons; within exon2, a single nucleotide polymorphism (rs1050450C > T) that results in a proline (Pro) to leucine (Leu) amino acid substitution at codon position 198 (Pro198Leu), close to the C-terminus of the protein, was observed. Several studies have reported that the presence of Leu at position 198 affects the binding of selenium, a necessary element for GPX1 function, to the enzyme and therefore decreases enzyme activity (9-11). Furthermore, the relationship between Pro198Leu polymorphism and cancer risk has been evaluated, and discrepant results were obtained. Some studies indicated a significant association of Pro198Leu polymorphism with breast cancer (12, 13), lung cancer (14, 15) and bladder cancer (16-20). In other studies, Pro198Leu polymorphism was not associated to breast cancer (21-23) and prostate cancer (19, 24-26).

In response to oxidative stress, a wide variety of stressed tumour cells show increased levels of GSTP1, and GSTP1 overexpression was found in many tumours such as esophageal cancer (27), colorectal cancer (28), renal cancer (29), lung cancer (30) and breast cancer (31-34). These studies have also demonstrated that high levels of GSTP1 correlated with cancer drugs resistance, failure of chemotherapy and poor prognosis of tumours. In the other hand, it was reported that loss of GSTP1 expression enhances cell susceptibility to acquire additional alterations and undergo further genetic changes toward tumour progression (35). In this field, several research works found lower GSTP1 expression in prostate cancer (36-40), endometrial cancer (41), hepatocellular cancer (42) and ovarian cancer (43). It has been shown that promoter hypermethylation is an epigenetic mechanism able to repress gene transcription by inhibiting the binding of transcription factors to their consensus sequences when methylated (44). In the studies cited above, GSTP1 down-regulation was associated to an aberrant methylation in the promoter region of GSTP1 gene (36-43). Moreover, *GSTP1* gene promoter methylation was detected in some body fluids of patients with prostate cancer and was proposed as a biomarker candidate for non-invasive detection of prostate cancer (45, 46). *GSTP1* gene is mapped to chromosome 11q13.2, spanning approximately 3 kb and comprising 7 exons and 6 introns (47).

The present study was planned to assess the presence of GPX1 Pro198Leu polymorphism and to evaluate GSTP1 expression –in relation to its promoter methylation status- in Moroccan population to determine whether they can be related to the risk of developing BC in Moroccan patients, thus to find a new biomarkers for better management of BC in Morocco.

Materials and methods

Study specimens

Before taking part in the study, informed consent was obtained from all patients, and this study was approved by the local institutional review board.

Cancer cases. 30 urinary bladder biopsies were collected from Urology department of the Military Hospital of Instruction Mohammed V in Rabat, Morocco. Tumor samples were collected by transurethral resection (TUR) or from cystectomy specimens for the routine diagnosis of BC. Each sample was divided into two similarly portions: one portion was formalin-fixed paraffin-embedded (FFPE) to be processed for histopathological examination in the Anatomopathology Department at the same hospital. Hematoxylin-eosin-stained sections were graded and staged according to World Health Organization (WHO) grading criteria and Tumour Node Metastasis (TNM) staging system; the other portion was stored at -80°C immediately after surgical removal for DNA extraction.

Control cases. 40 healthy peripheral blood samples collected at the National Blood Transfusion Center was used as controls for the analysis of GPX1 Pro198Leu polymorphism. One non-cancerous biopsy of BCG-treated patient and one normal urothelium tissue from a malignant specimen were recruited to be used as controls in the study of GSTP1expression and gene promoter methylation.

DNA extraction

Genomic DNA was extracted from fresh frozen tissue specimens and from blood samples using the Isolate II Genomic DNA Kit (BIOLINE), according to manufacturer's protocol, and stored at -20°C until use. DNA concentration and purity were assayed using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific).

GPx1 Pro198Leu genotyping

Pro198Leu polymorphism screening was carried out by PCR amplification and DNA sequencing (48). For PCR amplification, primers flanking a region in exon2 of GPX1 gene that contain the SNP rs1050450 C> T, GPX1-Ex2-F primer: 5'-CGCCACCGCGCTTATGACCG-3' and GPX1-Ex2-R primer: 5'-GCAGCACTGCAACTGCCAAGCAG-3' were used. PCR amplification was performed in a total volume of 25µL, containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200µM of each dNTP, 200nM of each primer, 0.25U Platinum Taq DNA polymerase (Invitrogen) and 100ng of genomic DNA. The mixtures were first denatured at 94°C for 7 min. Then, 35 cycles of PCR were performed with denaturation at 94°C for 30 s, primer annealing for 30 s at 60°C and primer extension for 30 s at 72°C. At the end of the last cycle, the mixtures were incubated at 72°C for 7 min. For every reaction, a negative control, in which DNA template was omitted from the amplification mixture, was included. PCR products were purified using the illustra ExoProStar 1-Step enzymatic clean up system (GE Healthcare Life Sciences), 1µl of 2X illustra ExoProStar 1-Step mix (Alkaline Phosphatase, Exonuclease I) was added to 6µl of PCR products, and the mixtures were incubated at 37°C for 15min, followed by incubation at 80°C for 15min to inactivate the enzymes. Sequencing of purified PCR products was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were performed in a final volume of 10µl, containing 1µl of 2.5X Big Dye ready reaction mix v.3.1, 10 pmol of forward primer and 100ng of purified PCR product. The mixtures were incubated at 96°C for 1min and 25 cycles were performed: denaturation at 96°C for 10s, primer annealing at 50°C for 5s and extension at 60°C for 4 min. The reactions were set to 30µl. To eliminate the excess of

labeled ddNTPs, sequencing reaction products were purified using sephadex G-50 gel-exclusion chromatography (GE Healthcare Life Sciences). Direct sequencing of amplified PCR products was performed on an ABI 3130xL Genetic Analyzer (Applied Biosystems). The sequences were analyzed using Sequence Scanner v2.0 software (Applied Biosystems).

Immunohistochemistry-based assessment of GSTP1 expression

Samples preparation. For immunostaining of GSTP1, FFPE biopsies were cut into 5µm sections, deparaffinised in xylene baths and rehydrated through a series of descending ethanol baths. After that, antigen unmasking was heat-induced using EnVision™ FLEX Target Retrieval Solution (Dako) at 99°C for 40 min, slides were then allowed to cool at room temperature. Endogenous peroxidases were blocked with EnVision™ FLEX peroxidase blocking reagent (Dako) for 5 min. IHC slides were incubated with anti-GSTP1 rabbit polyclonal antibody (Abcam) for 1 hour (diluted 1:500). Immunodetection was performed with En Vision™ FLEX HRP anti-rabbit secondary antibody and DAB peroxidase (HRP) chromogenic substrate (Dako), according to manufacturer's instructions. Slides were then counterstained with hematoxylin and dehydrated before microscopic examination.

Analysis of immunohistochemical staining. Examination of IHC-slides was done by light-microscopy by histopathologists. Cells with positive GSTP1 immunostaining were brown-stained. Two parameters were evaluated: intensity of immunostaining and percentage of positive stained tumor cells. The two parameters were separately assigned to a scoring system and were then combined in a total immunoreactive score (IRS). IRS was defined as the product of observed staining intensity (SI) and the percentage of positively stained cells (PP) (49). The details of the scoring system are shown in table I.

SI [0-3] *	**PP [0-4]** **	**IRS [SI×PP: 0-12]** ***
No staining = 0	No stained cells = 0	0-1 = negative expression
Weak = 1	< 10% = 1	2-3 = positive, weak expression
Moderate = 2	10-50% = 2	4-8 = positive, moderate expression
Intense = 3	51-80% = 3	9-12 = positive, strong expression
	> 80% = 4	

Table I. Details of immunoreactive scoring system (49)

Methylation Specific PCR analysis of GSTP1 gene promoter

Extracted DNA was converted with sodium bisulphite using EZ DNA Methylation™ Kit (Zymo Research). Briefly, 1µg of DNA was diluted in a final volume of 50µl containing 45µl of sterile water and 5µl of dilution buffer, 100µl of CT (Conversion Reagent) was added and the mixtures were incubated in the dark at 50°C for 12-16 hours. Modified DNA was then desulphoneted using desulphonation buffer and recovered in 10µl of elution buffer. For PCR amplification, 2µl of bisulfite-converted DNA was added to mixtures containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200µM of each dNTP, 200nM of each primer and 0.25U Platinum Taq DNA polymerase (Invitrogen), in a final volume of 25 µl. PCR reactions were carried out under the following conditions: first denaturation at 95°C for 2 min, 35 cycles of 30 sec 94°C, 30 sec 60°C, and 30 sec 72°C and a final extension for 7 min at 72°C. GSTP1 primers for unmethylated (U) and methylated (M) sequences are shown in table II (50). The PCR products were directly loaded onto a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination. DNAs extracted from MCF-7 and BCPAP cell lines were used as methylated and unmethylated controls, respectively.

Primer	Sequence	PCR product size
GSTP1 U/F	5'-GATGTTTGGGGTGTAGTGGTTGTTT-3'	97 pb
GSTP1 U/R	5'-CCACCCCAATACTAAATCACAACA-3'	
GSTP1 M/F	5'-TTCGGGGTGTAGCGGTCGTC-3'	91 pb
GSTP1 M/R	5'-GCCCCAATACTAAATCACGACG-3'	

Table II. Primers used for MSP of GSTP1 gene promoter (50)

Statistical analysis

Statistical tests were performed using the OpenEpi software. Chi-square test with Yates' correction was used to evaluate the association of GPX1 genotypes with the occurrence of BC and to examine the correlation between GPX1 genotypes and cancer stage or grade. The statistical relationship was considered as significant if the derived p-value was <0.05. The estimated genotypic and allelic frequencies were associated with 95% confidence intervals (CI) calculated using the modified Wald test (Agresti-Coull). The relationship between GSTP1 expression, BC clinical stage and tumour grade were analysed using Fisher's test. *p* values <0.05 were considered statistically significant.

Results

Histopathological data of cancer cases

Patients' age ranged from 42 to 78 years (mean age, 65 years), with the majority being male (n=26/30). At clinical staging, 3 patients (10%) had non-invasive tumours (Ta). Among invasive cases, 19 cases had non-muscle invasive disease (T1, 63%) and 8 of them had invasion of the muscle layer (T2, 27%). Histological grading revealed that 12 patients had low-grade cancer (40%) and 18 of them had high-grade disease (60%).

Detection of Pro198Leu polymorphism

All BC specimens and control samples were successfully amplified and sequenced; figure1 shows the obtained nucleotide sequences of GPX1 exon2. Sequencing analysis revealed rs1050450 C > T substitution resulting in Pro198Leu polymorphism in both BC specimens and controls; therefore the two genotypes Pro/Pro and Leu/Leu were found, while there was no Leu/Leu genotype. Obtained genotypic and allelic frequencies in both cancer cases and healthy controls are presented in table III. Statistical analysis showed no significant difference in the frequencies of Pro198Leu genotypes between cancer cases and healthy controls (p=0.425). Also, no significant difference was observed for the presence of Pro and Leu alleles in cancer and control groups (p=0.435). The distribution of Pro/Pro and Pro/Leu genotypes was also stadied according to stages and grades of cancer cases (table IV). Statistical analysis showed that there's any significant association between Pro198Leu polymorphism and cancer stage (Pro/Leu *vs* Pro/Pro: p=0.500) or tumor grade (Pro/Leu *vs* Pro/Pro: p=0.415); and then there was no significant difference in the distribution of alleles between clinical stages (Leu *vs* Pro: p=0.500) and grades (Leu *vs* Pro: p=0.427).

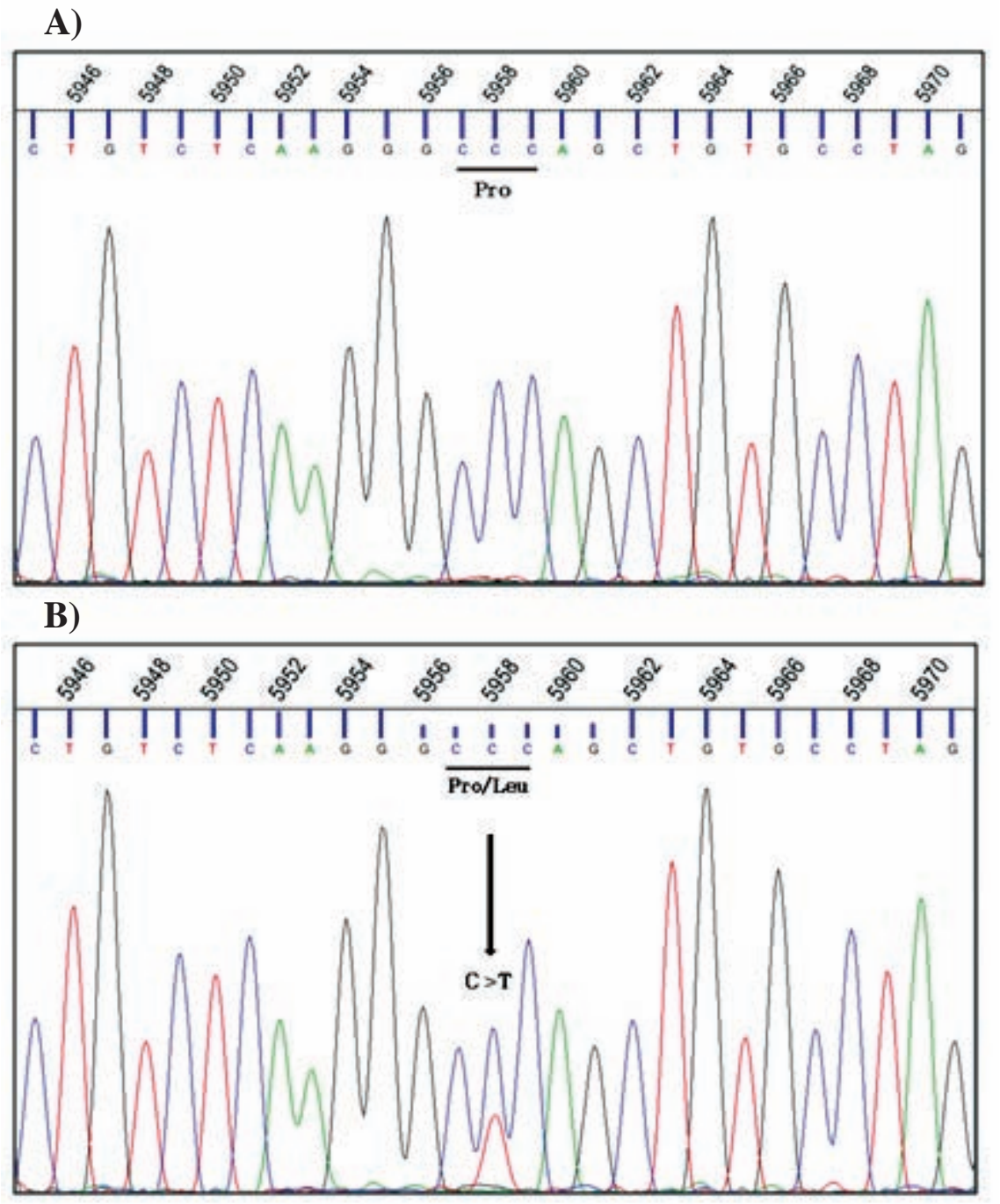

Figure 1. DNA sequence chromatograms of GPX1 exon2.
A: DNA sequence chromatogram of unaffected members; there is no C>T transition.
B: DNA sequence chromatogram of carries; there is a single base C>T transition in exon 2 that causes Pro198Leu substitution at heterozygote form.

Cases	Genotype		*p*	Allele		*p*
	Pro/Pro % [95%CI]	Pro/Leu % [95%CI]		Pro % [95%CI]	Leu % [95%CI]	
Bladder cancer	62.50 [45.21-77.12]	37.50 [22.88-54.79]	0.425	81.25 [69.87-89.09]	18.75 [10.91-30.13]	0.435
Controls	57.50 [42.18-71.5]	42.50 [28.5-57.82]		78.75 [68.49-86.38]	21.25 [13.62-31.51]	

Table III. Genotypic and allelic frequencies of GPX1exon2 in BC cases and controls

BC cases		Genotype		*p*	Allele		*p*
		Pro/Pro %[95%CI]	Pro/Leu %[95%CI]		Pro %[95%CI]	Leu %[95%CI]	
Stage	Ta-T1*	55.56 [33.7-75.46]	44.44 [24.54-66.3]	0.500	77.78 [61.67-88.53]	22.22 [11.47-38.33]	0.500
	T2**	66.67 [29.58-90.75]	33.33 [9.25-70.42]		83.33 [54-96.51]	16.67 [3.49-46]	
Grade	Low	55.56 [26.63-81.16]	44.44 [18.84-73.37]	0.415	77.78 [54.25-91.53]	22.22 [8.466-45.75]	0.427
	High	60 [54.25-91.53]	40 [8.47-45.75]		80 [62.33-90.86]	20 [9.14-37.67]	

* Ta-T1, grouped as early stages; ** T2, advanced stage.

Table IV. Genotypic and allelic frequencies of GPX1 exon2 according to clinical stage and grade

Analysis of GSTP1 expression by immunohistochemistry

GSTP1 expression was characterized histopathologically by brown staining and was found in both cell cytoplasm and nucleus. GSTP1 was expressed over the epithelium in benign and cancerous tissues with various GSTP1 staining intensities (figure 2). GSTP1 immunoreactivity was evaluated using the immunoreactive score (IRS) and results are displayed in table V. According to the IRS, samples were divided into groups with strong, moderate and weak GSTP1 expression. Over clinical stages of BC, the expression of GSTP1 was statistically evaluated and no significant association was obtained between GSTP1 expression and the invasion of cancer ($p > 0.05$). Among grades of cancer, variability in GSTP1 expression was also observed and evaluated, but statistically, it did not correlate with cell transition from low to high grade ($p > 0.05$).

GSTP1 promoter methylation

In MCF-7 cell line, used as methylation positive control, both unmethylated and methylated alleles were detected; while only unmethylated alleles were amplified in BCPAP cell line that was used as methylation negative control. *GSTP1* promoter appears unmethylated in all BC and non-malignant tissues. Indeed, none of the benign samples and the 30 cancer cases (all stages and grades mingled) showed methylated alleles, only the unmethylated alleles were detected (figure3).

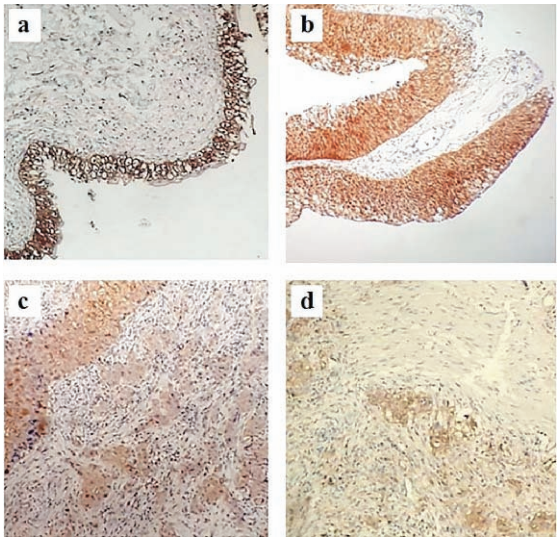

Figure 2. GSTP1 immunostaining in benign bladder urothelium and different status of BC (400× magnification). (a) Benign urothelial cells showing strong immunostaining. (b) Non-invasive tumour cells with strong immunostaining. (c) Chorion-invasive tumour cells with moderate immunostaining. (d) Muscle-invasive carcinoma displaying weak immunostaining.

GSTP1 immunoreactivity	Controls n (%)	BC cases						
		Stages			*p*	Grades		*p*
		Ta n (%)	T1 n (%)	T2 n (%)		LG n (%)	HG n (%)	
Strong (9≤ ISR≤13)	1 (50)	2(66.66)	4(21.05)	1(12.50)		4(33.33)	3(16.67)	
Moderate (4≤ ISR≤8)	1(50)	--	14(73.68)	4(50)	>0.05	7(58.33)	11(61.11)	>0.05
Weak (2≤ ISR≤3)	--	1(33.33)	--	3(37.50)		1(8.33)	3(16.67)	
Negative (0≤ ISR≤1)	--	--	1(5.26)	--		--	1(5.55)	
		3	19	8		12	18	
N	**2**	**30**				**30**		

Table V. GSTP1 immunoreactivity among benign cases and BC tumours according to stage and grade

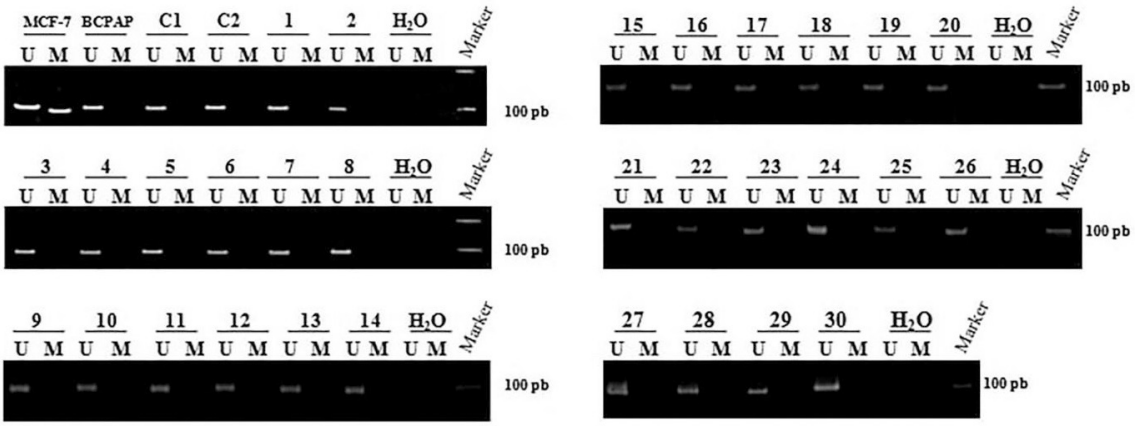

Figure 3. Results of MSP for GSTP1 promoter region.
Lanes U and M correspond to amplification with primers recognizing unmethylated (97bp) and methylated (91bp) sequences, respectively. DNA from MCF-7 cell line was used as positive control for methylation and DNA from BCPAP cell line was used as negative control. Results of normal bladder urotheliums (C1,C2: Control 1,2) and the 30 BC cases (1-30) are represented. Water was used as negative PCR control (H2O). On the right side: the 100 bp DNA ladder.

Discussion

During last decades, growing interest was given to the role of oxidative stress and antioxidant defense system in carcinogenesis. Glutathione Peroxidase-1 (GPX1) and Glutathione S-Transferase Pi (GSTP1) are two key antioxidant enzymes whose dysfunction has been well documented and reported in different types of cancers.

Accordingly, several studies have investigated the association of GPX1 Pro198Leu polymorphism, affecting GPX1 protein function, and cancer development, and the results were controversial depending on the study populations. A strong association was reported between GPX1 Pro198Leu polymorphism and breast cancer in Denmark and USA (12, 13); whereas other studies conducted in UK and USA didn't observe any significant association between Pro198Leu polymorphism and breast cancer among Caucasian women (21-23). Other studies suggested that Pro198Leu polymorphism is linked with risk of lung cancer (14, 15) but not with prostate cancer (19, 24-26).

Great interest was also given to the association between GPX1Pro198Leu and bladder cancer (BC) risk. Some studies have found that the polymorphism was associated with an increased

risk of BC. Ichimura et al. (16) showed that Pro/Leu genotype was significantly associated with BC in Japanese population (213 patients and 209 normal controls) (OR 2.63, 95% CI 1.45-4.75, p=0.001), and it was significantly correlated to advanced tumor stage (Ta-1 vs. T2-4, OR 2.58, 95% CI 1.07-6.18, p=0.034) but not with tumor grade. Furthermore, Paz-y-Miño et al. (17) found that Ecuadorian population with this polymorphism (97 cases and 120 controls) presented a probability of developing BC 3.8 times greater than controls (OR 3.8, 95% CI 2.16-6.78, $p< 0.001$) but the risk was higher with Leu/Leu genotype (52%) than Pro/Leu genotype (19%). Of particular interest, Kucukgergin et al. (18) have indicated that the Leu/Leu genotype of GPX1 was associated with a significantly higher risk of BC than the Pro/Pro genotype in Turkish population (157 patients and 224 healthy controls), and it was more frequently observed in BC patients with high-stage tumors than those with low-stage tumors. These findings were confirmed by two Chinese meta-analyses (19,20) suggesting that carriers of the variant T allele were associated with a significantly increased risk of BC (Leu vs. Pro, OR 2.111, 95% CI 1.020- 4.368, heterogeneity ($p< 0.001$); Pro/Leu and Leu/Leu vs. Pro/Pro, OR 1.876, 95% CI 1.011-3.480, heterogeneity ($p< 0.001$)).

In our study, we found no association of GPX1 Pro198Leu polymorphism with BC development (Pro/Leu vs. Pro/Pro: p=0.425; Leu vs. Pro: p=0.435) as well as no overall association with stages (Pro/Leu vs. Pro/Pro: p=0.500; Leu vs. Pro: p=0.500) and grades (Pro/Leu vs. Pro/Pro: p=0.415; Leu *vs*. Pro: p=0.427) of the disease. In spite of the relatively lower sample size of our study, our results are in agreement with previously reported data in Egyptian population (51) which was performed on 612 cases and 618 matched population-based controls, suggesting that the common genetic variation in GPX1 gene are not associated with overall BC risk (CT: OR 0.91, 95% CI 0.72-1.17; TT: OR 1.02, 95% CI 0.64-1.64). Interestingly, the Leu/Leu genotype, reported as highly aggressive form, was not found in our study.

These discrepant results could be attributed to the study population, with specific genetic aspects, and the sample size. Other potential confounding factors should be also considered including the technique used for point mutation analysis (e.g., RFLP may have a higher rate of false positives vs. DNA sequencing or Real-Time PCR assays) (51). Interestingly, some studies have reported that the combination of the GPX1 Pro198Leu polymorphism and other point mutations may have a synergistic effect on disease risk. In vitro functional analyses indicated that the combination of polymorphisms (Ala5/Ala6 and Pro198Leu) of the GPX1 gene had a 40% decrease in enzyme activity (48). Moreover, it was reported that gene-environment interaction can affect the genotype polymorphism. Of obvious interest, it would be to examine more polymorphisms and more genes along the same pathway, as well as non-genetic variables such as plasma antioxidant levels or lifestyle factors which affect oxidative stress, including cigarette smoking, consumption of alcohol and dietary antioxidants or supplements. Indeed, accumulating evidence has demonstrated that not all individuals having genetic alterations develop bladder cancer. Dif-ferences in the occurrence of bladder cancer among persons with the same GPX1 genotype may be attributable to carcinogen exposures and dietary factors that have been shown to increase carcinogen-derived oxidative radicals. Hansen et al. (52) studied whether the GPX1 Pro198Leu polymorphism and several lifestyle factors predict colorectal cancer risk. They observed a higher risk associated with alcohol consumption and smoking among homozygous GPX1 198Leu carriers, with incidence rate ratios for colorectal cancer of 1.45 (95% CI 1.17-1.81, p=0.02) per 10g alcohol intake per day and 2.56 (95% CI 0.99-6.61, p=0.02) among ever smokers compared with never smokers at enrolment.

Given its antioxidative and detoxification capabilities, GSTP1 protein was found to be expressed in most normal human tissues with a predominance in epithelial cells of respiratory, digestive and urinary tracts that are the most exposed systems to carcinogens (53,54). In comparison with

normal tissues, variability of GSTP1 levels was described in different human cancers. Some studies indicated an overexpression of GSTP1 in esophageal cancer (27), colorectal cancer (28), renal cancer (29), lung cancer (30), and in BC (54-56). Other studies showed reduced GSTP1 expression in prostate (36-40), endometrial (41), hepatocellular (42), and ovarian cancers (43). The results were controversial in breast cancer, so a number of studies found overexpressed GSTP1 (31-34) while others reported underexpressed GSTP1 (57,58).

In the present study, we evaluated the expression of GSTP1 by IHC in 30 BC biopsies and two noncancerous bladder tissues obtained from Moroccan patients. As expected, we detected GSTP1 protein mainly in cell cytoplasm; still, nucleic staining was also observed. The nuclear localization of GSTP1 has been described previously (32,54,59) its accumulation into the nucleus was ascribed to the overexpressed Bcl-2 protein that has been implicated as the regulator of transport of GSTP1 through the nuclear pore complex (55). Our immunohistochemical analysis demonstrated that GSTP1 is expressed in normal samples, showing strong or moderate expression and in most of the tumor cases, in which the expression was varying between strong, moderate, and weak. However, variant GSTP1 expression did not significantly correlate with the progression or malignant behavior of cancer ($p > 0.05$). Also, an inter-individual variability of GSTP1 expression in the normal samples and among patients with the same stage or grade of cancer was observed.

Because of the differential expression of GSTP1 in our specimens, we analyzed the methylation status of GSTP1 gene promoter region by MSP to verify its possible association with underexpressed GSTP1 cases. As a result, none of the normal bladder tissues or tumor samples expressed any methylated alleles. The methylation status of the promoter region of GSTP1 gene is controversial. In some studies, GSTP1 was found to be infrequently methylated and was not associated with grading or muscle invasiveness of urinary BC (50,60-62). However, Sacristan et al. (63) found that GSTP1 promoter was methylated in 44.6% of cases, but the methylation decreased with cancer progression, it classified Ta vs. T1 stages and distinguished LG vs. HG tumors. In addition, Casadio et al. (64) reported that GSTP1 methylation is capable of significantly predicting nonmuscle-invasive BC recurrence, its methylation frequencies were higher in nonrecurring than recurring tumors (26% vs. 5%) and were significantly indicative of a lack of recurrence at the 5 year follow up ($p = 0.02$) . All findings considered, the role of GSTP1 in the carcinogenesis of the bladder has yet to be defined.

Our finding of reduced GSTP1 expression in tumors not displaying methylated alleles was also reported in a study of Shilpa et al. (43) suggesting that promoter methylation may not be the only regulator mechanism in gene silencing; hence, other mechanisms may occur and affect GSTP1 transcription. Actually, transcription factors such as SP1, AP-1, NF-κB, and GATA1 were reported to play an important role in the regulation of GSTP1 expression (35). Similarly, Lo et al. (65) showed the ability of wild type p53 to transcriptionally activate GSTP1 gene, in that case, low GSTP1 protein level was associated with mutant p53. In the same way, Uchida et al. (66) demonstrated that GSTP1 expression may be repressed epigenetically by several miRNAs notably the miR-133a. Moreover, it should be noted that there is an individual variation of GSTP1 expression related to dietary and lifestyle factors (67).

Overall, this study is very informative: (a) GPX1198Leu mutant allele is rare in Morocco and the GPX1 Pro198Leu polymorphism alone cannot be associated to BC risk in our population; (b) GSTP1 is expressed in normal bladder tissues and in the majority of BC cases, showing a variation from intense to weak GSTP1 immunoreactivity; (c) the protein expression is not associated with disease' stage or grade; (d) GSTP1 downexpression found in some samples was not caused by gene promoter methylation.

Concusion

In conclusion, our results clearly showed no significant association between GPX1 Pro198Leu polymorphism and risk of bladder cancer (BC) in Moroccan population. Moreover, BC development seems to be not affected by GSTP1 expression, and therefore GSTP1 could not be used as a biomarker for BC management in Morocco. Also, variability in GSTP1 expression among BC cases was not due to GSTP1gene promoter methylation.

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EPIDERMAL GROWTH FACTOR RECEPTOR MUTATIONAL PROFILE IN LUNG CANCER - MOROCCAN COHORT

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Abstract

Background: Lung cancer constitutes the leading cause of cancer-associated mortality worldwide, with approximately 85% of lung cancer cases being non-small cell lung cancer (NSCLC) histological type. The study of epidermal growth factor receptor (EGFR) gene mutational profile in non-small cell lung cancer patients has a special clinical significance in the selection of patients for tyrosine-kinase inhibitor therapy. The aim of this study was to identify the frequency and spectrum of EGFR mutations in a cohort of Moroccan patients with lung cancer using the ADx-ARMS technology as routine method.

Materials and methods: We performed this study by processing 164 cases of NSCLC patients recruited between 2015 and 2018. Using the DNA extracted from the formalin-fixed paraffin-embedded FFPE tissue, we evaluated EGFR mutations using HRM-PCR, real time PCR "ADx-ARMS technology for results confirmation.

Results: The distribution of EGFR mutations in our cohort was as follows: 70% of patients having a deletion in exon 19, 10% in exon 21(L858R), 10% in exon 20 and 10% in exon 18 (G719X).
Conclusion: These results shows the need to incorporate the EGFR mutation test into routine practice and to develop rapid technological approaches in our laboratories for the availability of effective targeted therapy.

Keywords: NSCLC, ADx-ARMS, EGFR mutations, targeted methods, Moroccan cohort.

Introduction

Lung cancer constitutes the leading cause of cancer-associated mortality worldwide, with 2.1 million new lung cancer cases annually and 1.8 million deaths, representing 18.4% of the total cancer deaths. In Morocco, the incidence rates remain generally high (31.9 per 100,000) comparing to others countries in Africa [1]. There are two main types of lung cancer: The most predominant one is called Non-small cell lung cancer (NSCLC), which originates from bronchial epithelial-cell precursors and represents approximately 80-85% of all cases of lung cancer.

The second type is small cell lung cancer (SCLC), which originates from neuroendocrine-cell precursors, representing 15–20% [2]. The major form of lung cancer NSCLC, is classified into three histologic subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Adenocarcinoma all alone accounts for 38.5% of all lung cancers, with squamous cell carcinoma and large cell carcinoma accounting together for about 22.9% (20% and 2.9% respectively) [2, 3]. According to different parameters (stage, histological type and molecular characteristics) the treatment of lung cancer can include surgery, radiation therapy, chemotherapy, immunotherapy and/or target therapy [4] [5]–[8].

The etiology of lung cancer is reflecting the joint consequences of the interplay between exposures as etiologic agents and individual susceptibility to these agents. Despite the fact that the nature of these cancer drivers remains not fully determined, tobacco smoking represents the predominant cause of lung cancer. The accumulation of multiple genetic and/or epigenetic alterations, including those resulting in the activation of oncogenes and the inactivation of tumor suppressor genes is also one of the key factors characterizing Lung cancer initiation and progression [9]-[5]. A better understanding of the molecular mechanism by which these alterations affect lung cancer pathogenesis would provide new and more effective strategies for chemoprevention, early diagnosis, and targeted treatment [7].

Several driver genes were detected in lung cancer and the epidermal growth factor receptor (EGFR) represents the first oncogene identified in NSCLC [2] [10]. EGFR is a transmembrane glycoprotein consisting of an extracellular ligand-binding domain, a transmembrane domain, an intracellular TK domain, and a regulatory region. The tyrosine kinase (TK) receptor activates downstream RAS/RAF/MAPK, and PI3K/AKT signaling pathways, which cooperate to modulate several important mechanisms such as cell proliferation, adhesion, angiogenesis, migration, and survival. The activation of EGFR could be triggered by mutation or amplification/ over-expression causing upregulation of oncogenic cell signaling and malignant transformation [11], [12].

The discovery of EGFR gene mutations was one of the major breakthroughs in the study and investigation of the pathogenesis of NSCLC [13]–[15]. EGFR mutational profile in NSCLC patients has a special clinical significance in the selection of patients for targeted therapy and the approval of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) for the treatment of NSCLC represented a trend toward target therapy for advanced lung cancer patients [16]. The gefitinib and erlotinib as first generation EGFR-TKIs, have been demonstrated to significantly prolong the progression-free survival (PFS) time for patients with NSCLC and sensitive EGFR-mutations, primarily the exon 19 deletion (ex19del) or exon 21 codon 858 substitution (L858R) [5], [6]. The third-generation EGFR-TKIs, including AZD9291 and rociletinib, have demonstrated satisfactory efficacy in patients resistant to first generation EGFR-TKIs but only for patients with a second site mutation at codon 790 (T790M) in the EGFR exon 20 [17] **(Figure 1)**. However, the use of these targeted therapeutic agents requires EGFR screening of NSCLC patients to determine whether they should undergo EGFR-TKI treatment. In the last years, a large number of studies presented and recommended different methods for more efficient and quick identification of EGFR mutations in lung cancer patients, in comparison to the standard method 'direct sequencing'. Many of these methods and techniques are focusing only on the determination of the most frequent mutations and are called targeted methods. For this reason, several commercially available kits have been constructed; they provide quick and very sensitive results [18]–[22]. These methods have variable cost, and results turn-around time depends on their complexity and the sample flow.

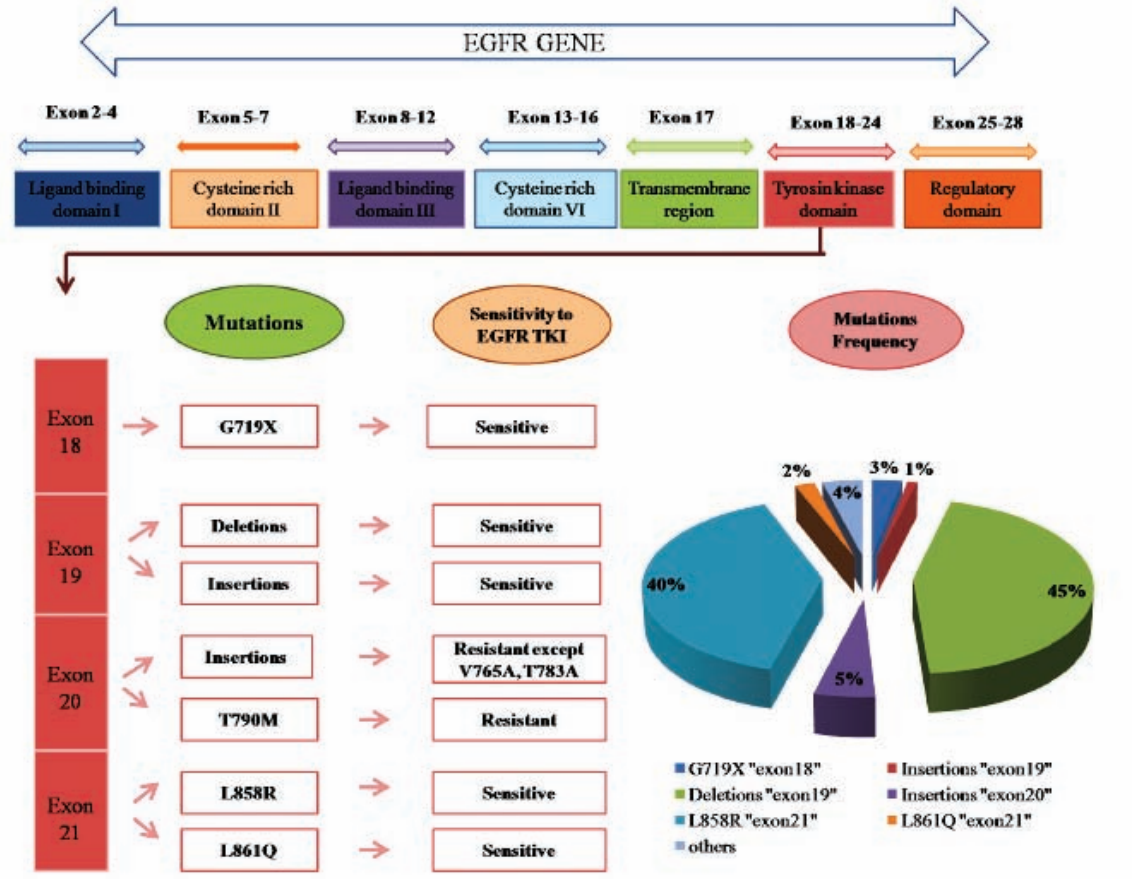

Figure 1: Epidermal growth factor receptor (EGFR) mutations in non-small-cell lung cancer (NSCLC). Exons location of the most common EGFR mutations, sensitivity to EGFR inhibitors "TKIs" and Frequency. TKIs: tyrosine kinase inhibitors « gefetinib, erlotinib and afatinib.

Materials and methods

Subjects: We performed this study by processing 164 cases of NSCLC patients recruited between 2015 2018. Informed written consent was obtained from all participants and approval from the local ethics committee was obtained.

DNA extraction: Genomic DNA extraction of the FFPE samples was performed with QIAamp DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. DNA concentration and quality were evaluated using a Nanodrop ultramicro spectrophotometer.

EGFR analysis: Using the DNA extracted from the formalin-fixed paraffin-embedded FFPE tissue, we attempted to identify somatic mutations in exons 18 to 21 of the tyrosine-kinase "TK" domain of EGFR gene. We evaluated EGFR mutations for 164 NSCLC patients using High Resolution Melt (HRM) polymerase chain reaction (PCR) and real time PCR **"ADx-ARMS technology** : AmoyDX Amplification Refractory Mutation System " for results confirmation.

Statistical analysis

All statistical calculations were performed using SPSS 24 for Windows software. Descriptive analysis was performed to provide a profile of the patient population. Continuous Variables were summarized by arithmetic means and standard deviation, whereas group categories were

expressed as percentages. Differences in mutation rates between groups were examined using the $\chi 2$ test, with statistical significance determined as p value less than 0.05.

Results and discussion

A total of 164 NSCLC patients were collected for this study. Most of our patients were males (68.9%) in the age range of 32-86 years old. The most represented histological type in the studied sample was adenocarcinoma (ADK) (89.6%) and for the smoking status 54.9% of the analyzed patients were smokers.

Among the positive mutant cases, distribution of mutations was as follows: 70% of patients having a deletion in exon 19, 10% in exon 21(L858R), 10% in exon 20 (6.7% T790M and 3.3% S768L) and 10% in exon 18 (G719X).

All of the positive patients with EGFR mutations were ADK and the frequency of never smokers in patients with tumors having EGFR mutations was significantly higher than that observed in smokers patients.

Table 1: Distribution of EGFR Mutation According to the patients Clinical parameters

Charactherisstics	EGFR mutations (n=29)	EGFR Wild Type (n=135)	pValue
Gender n (%)			**0.028**
Men	15 (51,7%)	98 (72,6%)	
Women	14 (48,3%)	37 (27,4%)	
Smoking Status n (%)			**0.043**
Yes	11 (37,9%)	79 (58,5%)	
No	18 (62,1%)	56 (41,5%)	
Hystological Type n (%)			**0.044**
Adenorcarcinoma (ADK)	29 (100,0%)	118 (87,4%)	
Squamous Cell Carcinoma (SCC)	0 (0,0%)	15 (12,6%)	

Previous studies have indicated that there is a variation in EGFR mutation distribution among different ethnicities. The results of the present study revealed that the EGFR mutation rate in Moroccan patients “17.8%” with NSCLC was lower than in Asian patients [23]–[26] and was higher than some white Caucasian patients [27] and trend to be lower than some white Caucasian patients [28], [29].

Comparing our results with those reported by a previous Moroccan study, we found that the EGFR mutation rate was decreased (21% vs 15.9%) and the difference founded between the two studies can be explained by the difference size [30].

Table 2: Variation of EGFR Mutation Rates in lung cancer Patients from different countries

Studies	Population	N° of cases	EGFR Mutation rate %	Screening method
[31]	Chinese (China)	858 ADK	327 (38.1%)	Direct Sequencing
[32]	Chinese (China)	219	111 (45.7%)	ARMS-PCR
[33]	Japanese (Japan)	277	111 (40%)	Direct Sequencing
[34]	Japanese (Japan)	437	165 (37.8)	Allele-specific amplification
[23]	Chinese (Taiwan)	101	39 (38.6%)	Direct Sequencing
[35]	Malaysian (Malaysia)	812	321 (39.5%)	Direct Sequencing & Real time PCR
[28]	African African American	67	12 (60.0%)	Sequenom mass array analyzer
[27]	White Australia	83	6 (7%)	Direct Sequencing
[29]	White Argentina	244	47 (19.3%)	Direct Sequencing
[29]	White Colombia	322	80 (24.8%)	Direct Sequencing
[29]	White (Peru)	381	119 (31.2%)	Direct Sequencing
[29]	White (Mexico)	203	136 (67%)	Direct Sequencing & ARMS Technology
[30]	North Africa (Morocco)	137	29 (21%)	TAQmAN &direct sequencing
	North Africa (Morocco) this study	239	38 (15.9%)	HRM, Real Time PCR «ARMS Technology» and IDYLLA
[36]	North Africa (Tunisia)	84	16 (19.04%)	real time PCR+NGS
[37]	Gulf region population	230	66 (28.70%)	quantitative polymerase chain reaction
[38]	Lebanese	106	9 (8.5%)	Multiplex real-time PCR
[39]	Jordanian	166	24 (14.7%)	PCR & Direct Sequencing

Studies have revealed that EGFR mutations are predictors of TKI treatment response and prognosis of patients with lung cancer. Clinical studies indicated that lung cancer patients harboring such alterations show a 70% to 80% response rate to TKIs [40]–[46]. Therapeutically, the mutations detected in our study population had clinical significance and the high frequency of exon 19 deletion founded in our cohort, shows that the ITKs drug could be more efficient for Moroccan lung cancer patients and consequently, they may have a better responsiveness rate.

These results shows the need to incorporate the EGFR mutation test into routine practice and to develop rapid technological approaches in our laboratories for the availability of effective targeted therapy. The targeted method presented in our study can be implemented as a simple and rapid method with a high specificity, sensitivity and simple data interpretation [47]–[52], comparing to direct sequencing who presents some limits demonstrated by previous studies: the low sensitivity, the requirement for large specimen size, the complexity and duration of the protocol, the high

cost and difficult interpretation of results [54], [55]. The HRM-PCR combined to real time PCR "ADx-ARMS technology" are very sensitive and specific methods, appreciated by the technical and medical team because it does not require high expertise in molecular biology and results interpretation.

In conclusion, given the rapid tumor progression in lung cancer, the analyze of the EGFR mutational profile after obtaining the anatomopathological results would ensure faster decision making about the most appropriate treatment strategy, which would be in the best interest of the patients.

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Abbreviations

ADK: Adenocarcinoma

ADx: AmoyDx

ARMS: Amplification Refractory Mutation System

EGFR: Epidermal Growth Factor Receptor

FFPE: Formalin Fixed Paraffin Embedded

HRM: High Resolution Melt

NSCLC: Non Small Cell Lung Cancer

PCR: Polymerase Chain Reaction

SCC: Squamous Cell Carcinoma

TK: Tyrosine Kinase

TKIs: Tyrosine Kinase Inhibitors

EXPRESSION PROFILE OF BREAST CANCER IN MOROCCAN WOMEN

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Abstract

Breast cancer (BC) is a major public health problem. Several transcriptomic studies have been conducted worldwide to elucidate breast carcinogenesis, but more are needed in developing countries like Morocco. This study was carried out to establish a genetic signature of breast cancer in Moroccan women.

Transcriptome profiling of 20 fresh breast tumors and normal matched adjacent breast tissues from Moroccan BC patients was analyzed using DNA microarrays.

1800 differentially expressed genes (DEGs) were identified: up-regulated genes were involved in mammary gland development, gene expression and signal transduction, while down-regulated genes were directly associated with regulation of kinase activity and signal transduction by protein phosphorylation.

This study is to our knowledge the first to analyze gene differential expression in Moroccan women. Focusing on the mRNA expression machinery may help to better understand gene interactions and explore new possibilities for BC targeted therapy.

Keywords: Breast cancer, Morocco, gene expression

Introduction

Breast cancer (BC) is one of the most common malignancies affecting the morbidity and mortality of women worldwide. Globally, more than 2 million BC cases were diagnosed and about 600.000 die from the disease every year, consisting of 25% of all cancers. In Morocco, BC is the first female cancer and the main cause of cancer related deaths [1,2].

Moroccan BC patients tend to present with an earlier age at diagnosis (53 years) compared to developed countries, more aggressive pathological type, higher grade tumor, advanced TNM staging, higher rates of lymph node involvement and higher proportion of triple negativity [3].

Epidemiological studies have suggested numerous risk factors including environmental and genetic factors [2].

In Morocco, only few biomarkers are included in the morphological classification and therapeutic orientation of BC. Based on gene expression analysis, BC could be subdivided into 4 distinct molecular classes: Luminal A, luminal B, Her2 positive, and basal like [4]. The systematic treatments supposed to eradicate the disease are delivered according to prognostic and predictive factors of the therapeutic response. Unluckily, they don't sufficiently take into consideration the evolutionary heterogeneity of the disease, frequently leading to unsuitable treatments. Therefore, other more accurate studies are needed in order to understand BC pathogenesis and to tailor therapeutic options for the Moroccan population.

In the present study, we analyzed the expression profile of BC representing the Moroccan population.

Materials and methods

Study population

20 fresh BC and normal adjacent breast tissues were collected from patients with clinically and histopathologically confirmed BC. Patients were recruited from Mohamed VI center for cancer treatment of Ibn Rochd University Hospital of Casablanca during 2016.

This study was approved by the Ethical Committee of Hassan II University, School of Medicine and Pharmacy, Casablanca, Morocco. A written informed consent was obtained from all participants before entering the study.

RNA isolation and gene expression analysis

In normal and BC tissues, we extracted the total RNA using the TRIzol® Reagent following the standard protocol. RNA quality and concentration were quantified using NanoVue TM Plus Spectrophotometer. RNA integrity was assessed by gel electrophoresis.

Gene expression profiling was performed using DNA microarrays. All data analyses were conducted using R specific packages.

Gene expression patterns were analyzed by advanced and open access bioinformatic tools.

Results and discussion

In the present study, we used DNA microarrays to determine differences in gene expression in 20 BC tissues compared to normal adjacent tissues, collected from a cohort of Moroccan BC females.

The mean age at diagnosis was 53 years old. Most patients had invasive ductal carcinoma. Our results showed that luminal B BC was the most frequent molecular subtype.

These results are in agreement with El Fatemi et al, which have reported that luminal B tumors are the most frequent molecular subtype in breast cancer of Moroccan women [5].

R software was utilized to identify the differentially expressed genes between BC tumors and normal adjacent tissues. Using an adjusted Pvalue<0.05 and a fold change >2/<-2. Among these, 800 were up regulated genes and 1000 were down-regulated genes in BC tumors compared to normal tissues **(Table 1**, **2)**.

To understand relevance and differences in genes expression patterns between BC tumors and normal tissues, Gene Ontology enrichment analysis was conducted. Up-regulated genes were involved in mammary gland development, organ morphogenesis, cell development, regulation of cell motility, and plasma membrane organization. While down-regulated genes, were mainly involved in apoptotic processes and regulation of kinase activity **(figure 1**, **2)**.

A number of genes aberrantly expressed between BC tumors and normal adjacent tissues were related to extracellular matrix, and metabolic pathways. In BC carcinogenesis genes involved in this pathways are subject to continuous remodeling, inhibiting and promoting tumor progression, according to stage of tumor development [6,7].

Table 1: Top five up-regulated genes involved in Moroccan BC women

Gene Symbol	logFC	FoldChange	P.Value
DLG3	2,04221419	4,11877177	2,70E-19
CCDC64B	2,63680428	6,21952449	1,10E-17
KRT19	3,06464878	8,36664246	1,40E-16
CREB3L4	2,39219945	5,24957072	1,29E-15
COL10A1	4,72540636	26,4538604	1,99E-15

Table 2: Top five down-regulated genes involved in Moroccan BC women

GeneSymbol	logFC	FoldChange	P.Value
ITGA7	-4,3515601	-20,4150345	1,55E-22
PKDCC	-3,29786049	-9,83455991	2,33E-22
EHD2	-2,7254612	-6,61371647	3,78E-22
GPAM	-4,16902649	-17,9887931	1,09E-21
LPL	-5,50963273	-45,5580069	1,27E-21

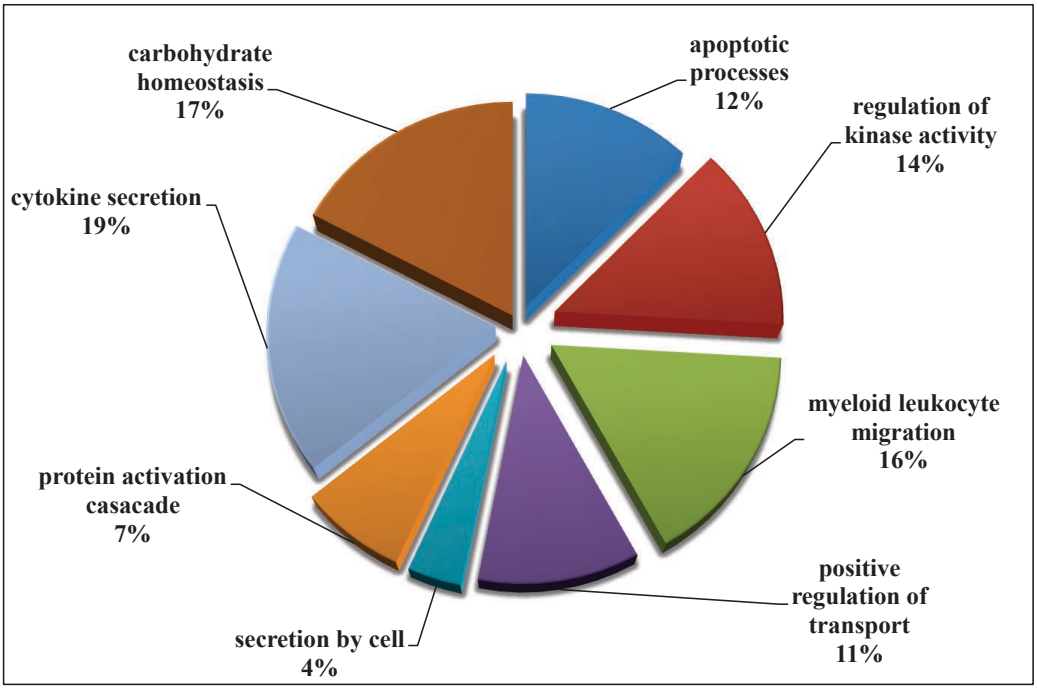

Figure 1: Biological Process of up-regulated genes involved in Moroccan BC women

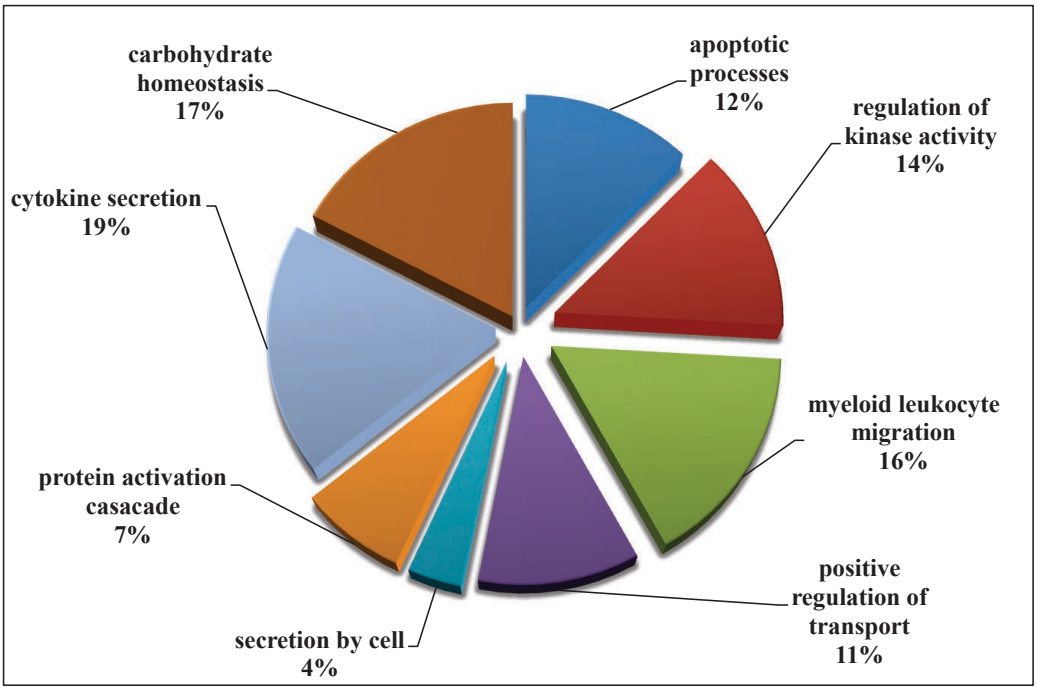

Figure 2: Biological Process of down-regulated genes involved in Moroccan BC women

Conclusion

Our study was the first to analyze the expression profile of breast cancer representing the Moroccan women. These results provide useful information for exploring potential novel biomarkers as diagnosis and therapeutic targets to improve the clinical treatment of BC in Moroccan population.

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N.B. These results are part of an already submitted paper

COMPARATIVE STUDY OF CLINICOPATHOLOGICAL FEATURES AND PROGNOSIS IN TRIPLE NEGATIVE AND NON-TRIPLE NEGATIVE BREAST CANCER IN THE NORTH OF MOROCCO

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Abstract

Breast cancer is a heterogeneous disease that can be classified into diverse subtypes with distinct biology and prognosis. The purpose of this study is to compare clinicopathological features and prognostic of patients with Triple Negative Breast Cancer (TNBC) and non-TNBC. Clinicopathological features and prognosis of 266 patients from north Morocco (56 TNBC and 210 non-TNBC) were evaluated using SPSS 20 software. The incidence of TNBC was 21%. Compared with non-TNBC, TNBC patients tend to be younger at diagnosis and had slightly larger tumors and higher stage. Higher histological grade was strongly associated with TNBC. Lymph nodes and histological type were similar in the two groups. Bone was the most frequently metastatic site in all breast cancers, but TNBC was strongly associated with liver metastases.

No significant difference was observed in 5-year Disease-Free Survival (DFS) and 5-year Overall Survival (OS) between TNBC and non-TNBC. In conclusion, TNBC is associated with particular clinicopathological features and poor prognosis compared to non-TNBC.

Keywords: Triple negative breast cancer**,** Non-triple negative breast cancer, Clinicopathological features, Prognosis, North of Morocco.

1. Introduction

Breast cancer is a heterogeneous disease comprising many various entities that have diverse pathological, prognostic and molecular features. Based on gene expression profiling, breast cancer is classified into several molecular subtypes: luminal A, luminal B, ERBB2, basal-like and normal-like [1, 2]. Based on the expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 expression, breast tumors can be classified into three subtypes using immunohistochemical

methods: luminal, HER2+ and Triple Negative Breast Cancer (TNBC). Compared with other subtypes, TNBC is generally more aggressive and have usually a worse prognosis [3-5]. The aim of the present study is to evaluate differences in clinicopathological features and prognostic of TNBC and non-TNBC subtypes in a cohort of north Morocco.

2. Methods

2.1. Study population

We reviewed data from Oncology Clinic Al Amal of Tangier and we identified 331 patients with invasive breast cancer diagnosed between January 2010 and December 2015. We excluded patients in whom ER, PR, and HER2 status were not available. The present study involved 266 patients, among them, 56 had a TNBC, and 210 had a non-TNBC. Our study was approved by the Ethics Committee for Biomedical Research in the Faculty of Medicine and Pharmacy of Rabat (CERB) and informed consent was obtained from all patients.

2.2. Pathological data

ER, PR and HER2 status were determined using immunohistochemistry (IHC). ER and PR were classified as negative when less than 1% of the tumor cells demonstrated positive nuclear staining. For HER2, negative status was defined as scores of 0 and 1+ and receptor over-expression with scores of 2+ that are lacking HER2 gene amplification detected by FISH. Breast cancer with ER, PR, and HER2 classified as negative was defined as TNBC. The remaining cases were defined as non-TNBC.

2.3. Statistical analysis

Patient characteristics were compared between TNBC and non-TNBC using the $\chi 2$, the t Student or the Mann-Whitney tests. A *p-value*<0.05 was considered statistically significant. Survival outcomes were estimated using Kaplan-Meier method and differences were tested for statistical significance using the log-rank test. All statistical tests were performed using SPSS 20 software.

3. Results

3.1. Clinicopathological characteristics

A total of 266 patients diagnosed with invasive breast cancer were enrolled in this study, 56 (21%) exhibited a TNBC and 210 (79%) exhibited a non-TNBC. The Median age at diagnosis for TNBC *vs*. non-TNBC patients was 46 years *vs*. 49.5 years respectively (*p-value*=0.147). The distribution of patients according to age showed that TNBC affects younger women than non-TNBC (**Figure 1**), but the difference observed was not statistically significant (*p-value*=0.628). The TNBC pre-menopausal patients accounted for 35.7%, which was not different from the non-TNBC group.

In the TNBC group, 57.1% of patients were diagnosed with early breast cancer (I, II) and 42.9% were diagnosed with advanced breast cancer (III, IV), which was a distribution similar to that of the non-TNBC group (*p-value*=0.967). High frequencies of not otherwise specified histological type were observed in both TNBC and non-TNBC group, other histological special type including invasive lobular carcinoma and medullary carcinoma were also observed in the two groups. The tumor grade III was most commonly associated with TNBC (*p-value*=0.002). In the non-TNBC group, we have observed an increase in node positivity as tumor size increased, 78.9% of patients with positive nodes had large tumor size (> 5cm), This correlation was statistically significant

(*p-value*=0.003). Among the TNBC patients, no correlation between tumor size and node status was observed (*p-value*=0.305). The comparison of all clinicopathologic features of TNBC and non-TNBC patients are shown in **table I**.

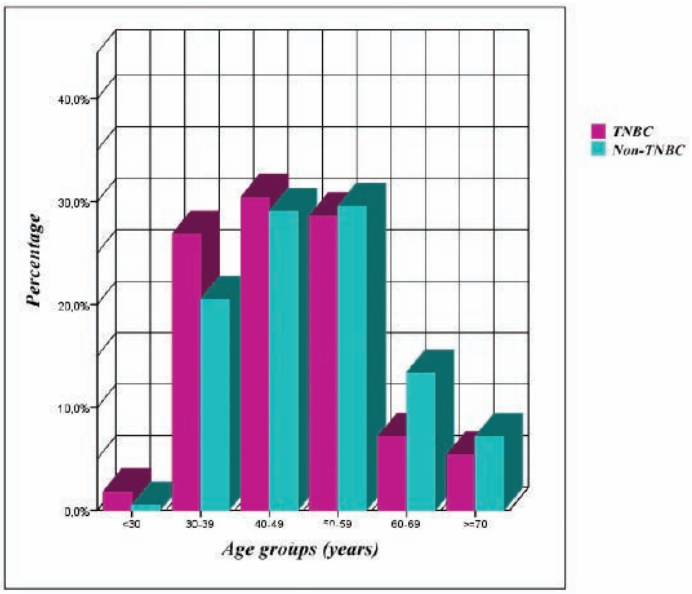

Figure 1: Distribution of TNBC and non-TNBC patients by age group

Table I: Comparison of clinicopathological features of TNBC and non-TNBC patients

Variable	TNBC	Non-TNBC	P-value
Median age at diagnosis	46 years	49.5 years	0.147
Menopause			0,057
Yes	35.7 %	50 %	
No	64.3 %	50 %	
TNM stage			0.967
I	8.9 %	9.5 %	
II	48.2 %	50.9 %	
III	25 %	24.8 %	
IV	17.9 %	14.8 %	
Tumor size			0.292
<2 cm	16.1 %	22.9 %	
2-5 cm	73.2 %	61.9 %	
>5 cm	10.7 %	15.2 %	
Lymph nodes status			0.132
N-	57.9%	44.3 %	
N+	42.1%	55.7 %	
SBR grading			0.002
I	1.8 %	14.8 %	
II	50.9 %	58.6 %	
III	47.3 %	26.7 %	
Histological type			0.206
IDC-NST	92.9 %	87.7 %	
Other	7.1 %	13.3 %	

(IDC-NST: Infiltrating Ductal Carcinoma – No Special Type)

3.2. Recurrence and metastasis

In the TNBC group, 19.6 % of patients developed recurrence and metastasis. However, for patients with non-TNBC recurrence occurred in 16.2 % of patients (*p-value*=0.587). The average time to recurrence was 26 and 27 months in TNBC and non-TNBC respectively (*p-value*=0.929). Common distant metastatic sites were the bone, liver, lung and brain. Compared with non-TNBC, TNBC patients exhibited a higher proportion for liver metastasis (56.3 *vs.* 23.6 %, *p-value*=0.013). These findings are summarized in **Table II**.

Table II: Comparison of recurrence or metastatic status of TNBC and non-TNBC patients

Characteristics	TNBC	Non-TNBC	P-value
Metastatic or recurrence	19.6%	16.2%	0.587
Metastatic site			
Bone	62.5%	70.9%	0.522
Lung	43.8%	30.9%	0.339
Liver	56.3%	23.6%	0.013
Brain	12.5%	3.6%	0.176

3.3. Survival analysis

5-year Disease-Free Survival (DFS) rate of no-metastatic TNBC and non-TNBC patients were 80.4 and 83.8%, respectively (*p-value*=0.379). 5-year Overall Survival (OS) rate for all TNBC and non-TNBC patients were 75 and 78.1%, respectively (*p-value*=0.355) (**Figure 2 and 3**).

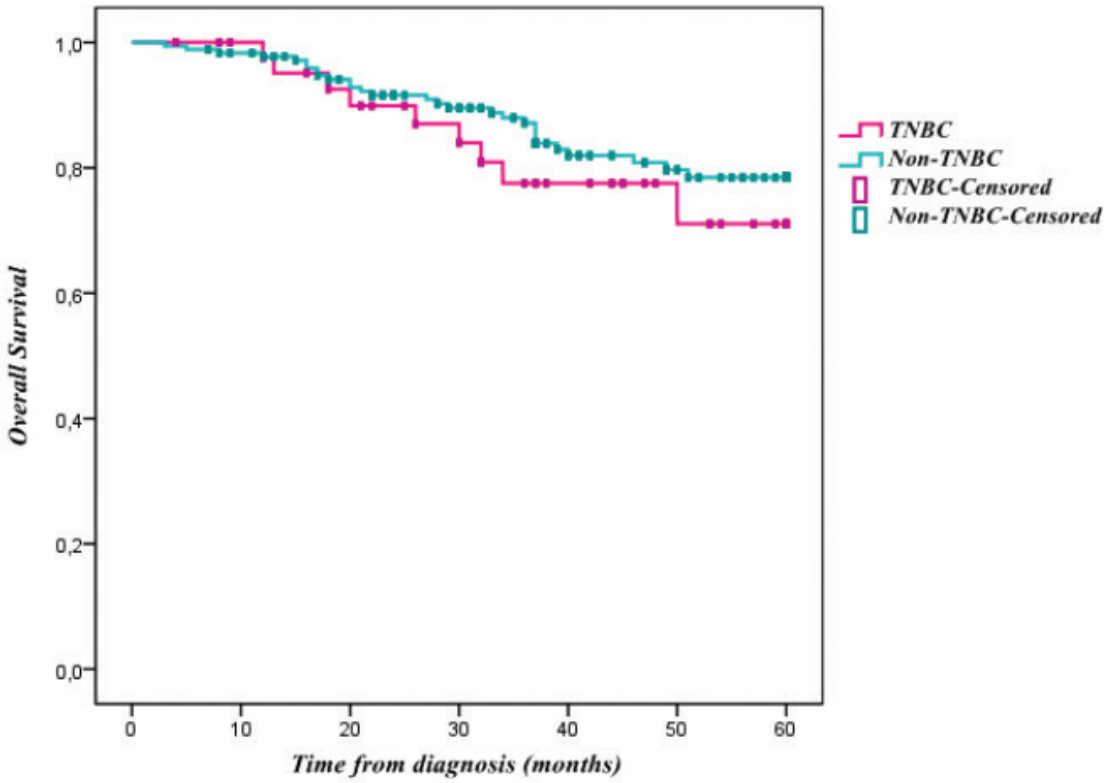

Figure 2: Comparison of disease-free survival at 5 years of TNBC and non-TNBC patients

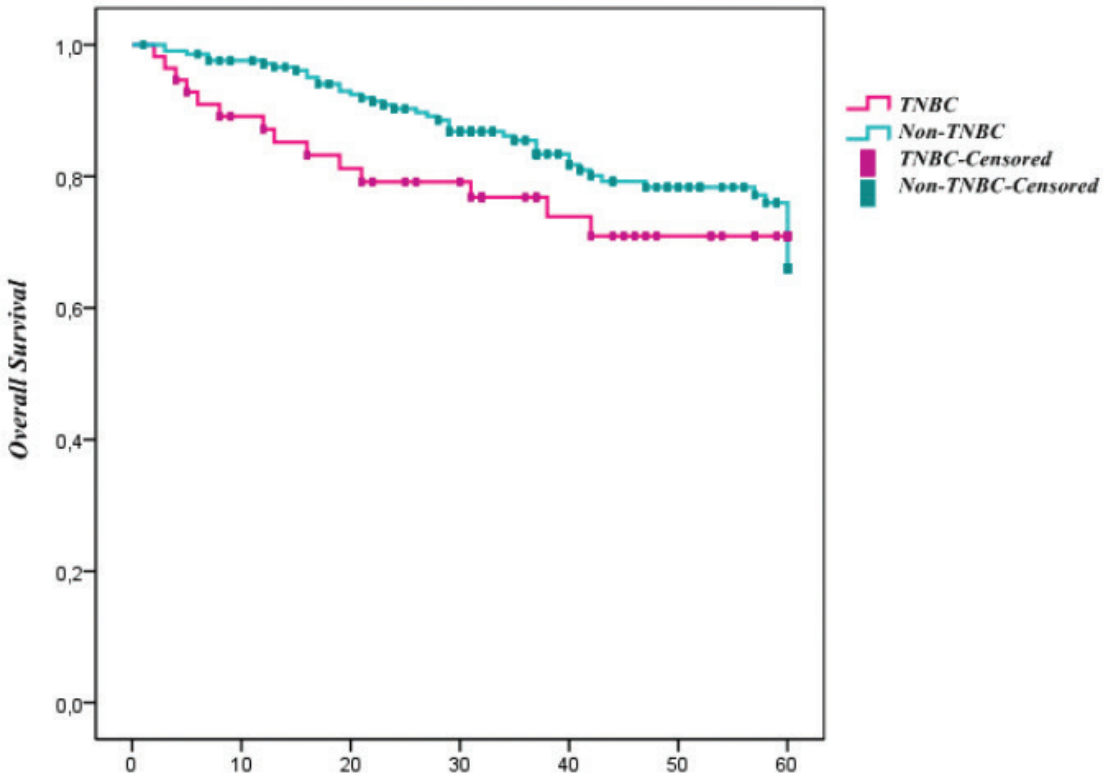

Figure 3: Comparison of overall survival at 5 years of TNBC and non-TNBC patients

4. Discussion

Breast cancer is a multifaceted disease, their diverse subtypes are associated with varied behaviors and outcomes. Compared with other types of breast cancer, TNBC has distinct clinicopathological features, which are associated with a poor prognosis. Several comparative studies have demonstrated that TNBC is more common among young and premenopausal women [6-8]. In our study, more than half of TNBC patients (59%) was under 50 years of age and approximately two-thirds (64.3%) were premenopausal at the time of diagnosis, However, there is no significant difference in onset age between TNBC and non-TNBC patients.

TNBC patients are more frequently presented with large tumor size (>2 cm) than non-TNBC patients [3, 9, 10]. In terms of node status, comparative studies between TNBC and non-TNBC have provided contradictory results [3, 11, 12]. Larger breast tumors tend to be associated with a greater number of axillary lymph nodes involved with metastatic tumor than smaller tumors [13], but, this is not the case in TNBC. Among TNBC, there was no correlation between tumor size and node status, however, this correlation exists in the non-TNBC group [3]. In our study, we observed a strong correlation between tumor size and node status in the non-TNBC group (*p-value* = 0.003). Although, this correlation was not found in the TNBC group (*p-value* =0.305). Compared with non-TNBC, TNBC was correlated with higher histological grade, those results were consistent with previous findings [3, 9]. We have reported that the TNM stage and the histological type of TNBC were similar with non -TNBC.

TNBC is associated with a particular recurrence pattern. Dent *et al.* have reported that the recurrence and death occurred in the first five years after diagnosis, especially in the first three years after

diagnosis. The risk of any recurrence dropped quickly thereafter. In the other group, the risk of recurrence is low within 5 years of diagnosis, however, distant recurrences continued to accrue for up to 17 years after diagnosis [3]. These findings were confirmed by further studies [10, 14]. In our study, we did not found an increased risk of recurrence in the TNBC group. Visceral organs and brain are the most common sites of recurrence in TNBC, the non-TNBC tend to metastasize to the bone [4]. Our results have demonstrated that bone was the most common metastatic site in all breast cancers, furthermore, TNBC was strongly associated with liver metastases. It has been proposed that preferential tumor cell at certain organ may be related with the expression of specific genes [15, 16]. The prognosis of TNBC, compared non-TNBC, is definitely pejorative, in terms of DFS and OS. Studies showed that the rate of DFS and OS in TNBC group was lower than the non-TNBC group [3, 17, 10]. No differences were observed when we have compared DFS and OS rates in TNBC and non-TNBC groups. Authors have suggested that the reason behind the poor prognosis of TNBC may be related to their special clinicopathological characteristics at the time of diagnosis and the unavailability of targeted therapy [18, 19].

5. Conclusion

TNBC is associated with particular clinicopathological features and poor prognosis compared to non-TNBC. In north Morocco, a larger cohort is needed to identify specific characteristics of these two groups. A better understanding of behavior of TNBC and non-TNBC is interesting for the development of new target therapies and the improvement of the prognosis.

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