PhD Thesis

'POSSIBILITIES AND LIMITS OF REAL TIME PCR TECHNIQUE IN CUTANEOUS NEOPLASIA'

ABSTRACT

Scientific Coordinator

Prf. PhD. Luminița Smaranda IANCU

PhD student

Elena ANDRESE (PORUMB-ANDRESE)

Iași

2016
<table>
<thead>
<tr>
<th>CONTENTS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>General Part.</td>
<td>2</td>
</tr>
<tr>
<td>Introduction.</td>
<td>2</td>
</tr>
<tr>
<td>Motivation of Study.</td>
<td>3</td>
</tr>
<tr>
<td>Structure and Function of the Cutaneous Organ.</td>
<td>5</td>
</tr>
<tr>
<td>Ocurrence of cutaneous neoplasia.</td>
<td>9</td>
</tr>
<tr>
<td>Risk Factors.</td>
<td>13</td>
</tr>
<tr>
<td>The impact of cutaneous neoplasia on the quality of life.</td>
<td>15</td>
</tr>
<tr>
<td>Clinical forms.</td>
<td>19</td>
</tr>
<tr>
<td>Pathogenesis.</td>
<td>24</td>
</tr>
<tr>
<td>Diagnosis.</td>
<td>30</td>
</tr>
<tr>
<td>Treatment.</td>
<td>38</td>
</tr>
<tr>
<td>Prophylaxis of cutaneous neoplasia.</td>
<td>42</td>
</tr>
<tr>
<td>PERSONAL PART.</td>
<td>45</td>
</tr>
<tr>
<td>Retrospective study regarding epidemiological data of cutaneous neoplasia</td>
<td>46</td>
</tr>
<tr>
<td>Materials and method.</td>
<td>46</td>
</tr>
<tr>
<td>Results.</td>
<td>48</td>
</tr>
<tr>
<td>Discussions.</td>
<td>61</td>
</tr>
<tr>
<td>Conclusions.</td>
<td>64</td>
</tr>
<tr>
<td>Prospective study regarding the presence of the BRAFV600E mutation in the cutaneous melanoma</td>
<td>65</td>
</tr>
<tr>
<td>Objectives of the study.</td>
<td>65</td>
</tr>
<tr>
<td>Material and method.</td>
<td>68</td>
</tr>
<tr>
<td>Establishing study groups.</td>
<td>73</td>
</tr>
<tr>
<td>The processing of the paraffin embedded tissue blocks.</td>
<td>87</td>
</tr>
<tr>
<td>DNA extraction.</td>
<td>89</td>
</tr>
<tr>
<td>Detection of BRAFV600E mutation.</td>
<td>98</td>
</tr>
<tr>
<td>Results.</td>
<td>100</td>
</tr>
<tr>
<td>Discussions.</td>
<td>110</td>
</tr>
<tr>
<td>Conclusion for prospective study.</td>
<td>118</td>
</tr>
<tr>
<td>FINAL CONCLUSIONS.</td>
<td>119</td>
</tr>
<tr>
<td>PERSPECTIVES.</td>
<td>121</td>
</tr>
<tr>
<td>LIST OF PUBLISHED PAPERS.</td>
<td>122</td>
</tr>
<tr>
<td>ANNEXES.</td>
<td>123</td>
</tr>
<tr>
<td>BIBLIOGRAPHY REFERENCES.</td>
<td>124</td>
</tr>
</tbody>
</table>
The PHD Paper is illustrated by 64 figures and 28 tables and contains 267 bibliographic references. This summary selectively presents the iconography and bibliography from the text, following the numbering and contents from the paper in great detail.

1 THE GENERAL PART

1.1 Introduction

Modern medicine brings major changes in the way we approach the cutaneous neoplasia, in the way we understand its etiopathogenesis as well as in the way we treat a patient with this diagnosis. At present the tendencies head towards the study of the individual. Each action is perceived from the point of view of the patient and this confirms again that 'there is no disease but ill people'.

The concept of 'personalised medicine' is based exactly on the personal differences and tries to develop new study techniques of the different parts of the genome of even the whole genome itself. Through this concept we do not try to find strictly personalised therapeutic solutions, but we try to divide the population into sub-groups according to each individual's sensitivity to develop a certain illness or having a response to a certain therapeutic operation, this selection being based on the different changes of the DNA. This concept was introduced for the first time by Gibson and tends to define itself as a distinctive branch of medicine by supporting more and more fields like molecular biology or genetics. These two fields have developed very fast in the last decades. At the same time, the growing interest in this concept is supported by the fact that, only some medicine has 100% effect and many of the therapeutic operations should be tailored for the individual (1).

Cutaneous neoplasia is a multifactorial entity, and the genetic factor plays an important role in its etiopathogenesis. This is done by identifying the cutaneous phenotype or association of various syndromes with susceptibility towards neoplasia (e.g. Li-Fraumeni, xeroderma pigmentosum, basal cell nevus, syndrome of familial neoplastic nevi) or by changes which took place in genes (3).

Following the raise of life expectancy at a global level and the occurrence of non-melanocytic neoplasm skin cancer (Non Melanoma Skin Cancer) is rising. It is estimated that until 2030, at a global level, the number of cases could be 50% higher (4).

Due to the fact that classifications of cutaneous neoplasia based on morphopathological criteria have not generated relevant information for finding promising therapy in this field, in the present paper we aim to point out the changes which take place at the DNA level by using a molecular biology technique. We chose a gene whose mutation has a clinical/prognostic significance for the evolution of the diagnosed patient suffering from one of the most aggressive form of cutaneous neoplasia - malignant melanoma. From the variety of present studied genes in dermato-oncology, there is therapeutic option for only one of them. This is validated by
specialised studies approved by FDA (Food and Drug Administration) and it is available in Romania too - called Vemurafenibum.

This way, we chose to point out BRAFV600E mutation in patients with malignant melanoma because of more reasons: melanoma is an aggressive form of cutaneous neoplasm with a very high mortality rate. In the last 3 decades, the positive effects of this therapy on this type of neoplasm have had minim success. In more advanced cases (III-IV) alternative therapy options such as BRAF inhibitors are among the most promising. The identification of this type of change at DNA level classifies patients in eligible or non-eligible for therapy with Vemurafenibum (Zelboraf®). For the eligible patients there is possibility to improve their survival rate (5-8).

1.2 Motivation of study

The proposed topic is part of the tendencies of fundamental as well as applicative and present science research which aims to define a general profile of this type of neoplasia. The chosen idea for research is developed up to a molecular level, with the help of RT-PCR reaction. Its interdisciplinary character, dermatology-microbiology, brings a genuine aspect to the study.

The fact that mutations of different genes are found in the majority cancer cells means that these genetic changes are subject to studies which propose new alternatives for therapy. The results we had in this study allowed us to compare them to data published in specialised literature. This is the first study of this type done in the North-East part of Romania.

The main motivation for this study has been the need to change the way to approach cutaneous neoplasia, approach that regards more and more data from molecular biology in stage diagnosis and treatment of the neoplasia patient. Data obtained from the different stages of testing DNA emphasise a new tendency in medicine- the personalised medicine. We chose the melanoma because this entity has an aggressive evolution; it riches the metastasis way ahead and management of these cases have not presented any major improvement in the recent years.

The chose topic aims to contribute to the attempts to search an optimum inhibitory therapy against the genetic mutations up to present. This concept hopes to find solutions for clinical problems with the help of paraclimical methods of research as instrument for efficient therapy solutions.
1.8 Pathogenesis of cutaneous neoplasia

Although most authors conclude that the pathogenesis of cutaneous neoplasia is a multifactorial one, most of them accept the important role that UV rays play in the case of melanoma as well as in NMSC (79) case.

Another very well-known aspect is the fact that the neoplastic process appears due to genetic mutations which alters the cellular proliferation, differentiation and death. These mutations are found in three distinctive gene categories: proto-oncogene, tumour suppressor genes, and DNA repairing genes. Any mutation at the level of any gene can have as consequence the introduction of a neoplastic process (80).

The tumour suppressor genes balance the normal cellular growth and their differentiation. The best known gene of this type which is involved in the pathogenesis of cutaneous neoplasia is p53 gene. Changes at this level have been directly linked to the neoplastic process, approximately 50% of cancer cases. This gene is also known as 'the guardian of the human genome' due to its functions of cellular cycle balancing, conservation of genic stability as well as prevention of mutations. Additionally, the protein which is coded by this gene can block the process of tumoral angiogenesis which appears as a response to DNA alteration: DNA ruptures, genic overexpression or activation of some oncogenes (81). It is interesting that mutant p53 protein, not only that it loses its function to tumoral suppression but it develops new functions: promotion of proliferation of neoplastic cells, anti-apoptosis, angiogenesis, promotion of metastasis or metabolic changes (82, 83).

Among the genes proven to have a role in etiopathogenesis of melanoma in humans, ER (estrogen receptor gene) has only recently started to be studied following observations that revealed a lower MM occurrence in women than men. This way it was emphasized that patients with MM metastasis have a lower expression ER β at the level of tumoral tissue. This suggests a possible role of the receptor in the metastasis process (84). Other genic changes connected to cutaneous neoplasia are: WNT, Ras, p16INK4 (cyclin-dependent kinase inhibitor 4), NF-Kb, Kit or c-Myc.

Ras oncogene is another mutant gene in skin neoplasia. The three Ras genes that have been discovered until present (Harvey-Ha, Kirsten –Ki and N-ras) are considered to be the most frequent oncogenes present in the human cancer. Among these, N-ras could be the one which contributes the most to this neoplastic process with cutaneous localization. Present data associates this oncogene with UV-induced lesions or with xeroderma pigmentosum (85-87).

WNT gene codes a group of approximately 19 glycoproteins rich in cysteine with plays the role of ligands in order to activate the signalling paths receptor-mediated, paths which control the cellular differentiation, proliferation and motility (88).

Kit is another important oncogene associated with cutaneous melanoma, gene that codes a receptor trans-membranal of tyrosine kinesin. Mutations of Kit occur in 10-15% of cases of acral and mucous melanoma and less in lesions from photo-exposed areas (89-91).
At the present antineoplastic therapy is based on chemotherapeutic agents that destroy the cancer cells as well as the non-cancer cell. Recent trends to find alternatives at DNA level aim towards pathological changes at molecular level and this way they reduce toxicity towards the normal cell in the body.

The role of UV rays in the pathogenesis of this type of cancer has been acknowledged since 1894. It is considered that this factor brings important changes at molecular level. Changes at the level of p53 gene are considered to be 'the signature' of UV rays left on the human DNA (fig.1.3) (92).

UV radiations have been considered as a risk factor of cutaneous cancer for a long time due to the multiple effects which they have on the skin. These effects contribute a great deal to the development of neoplasia by: modifying the DNA, inducing the immunosuppression or facilitating oxidative stress.

The neoplasia that occurs on the photo-exposed areas (head, neck) is one of the most aggressive types, with a rate of local reoccurrence which reaches approximately 47% (93).

The UV radiations are divided into 3 big categories, according to the wave length: UVA (320-400 nm), UVB (280-320 nm) and UVC (100-280 nm). UVA are capable to go beyond the stratosphere, reaching the Earth in 90-99% of cases. These rays of low energy are capable to deeply penetrate the skin due to the big wave length and lead to appearance of reactive types of oxygen which denature the DNA. However, they lead to cancer in a smaller proportion than UVB rays.

Figure 1.3 The effect of UV rays in inducing cutaneous carcinoma (92)
UVB radiations reach the Earth in proportion of 1-10% and are a hundred times more mutagenic than UVA radiations. Their effects are located at the level of superficial layers of the skin due to the wave length and they present the following: erythema, hyperpigmentation, sunburn, premature ageing of the skin last but not least carcinogenesis (94).

UVC has minor effects on the skin due to the filtration done by the atmosphere.

The effect of carcinogenesis is made by lowering the local immunosuppression. This effect is produced by more mechanisms: decreasing the level number of antigen presenting cells in skin as well as of Langerhans cells, or releasing proinflammatory mediators (TNFα, IL-10) (95). Although the exposure to type B UV rays has been directly connected to inducing changes of p53 expression in skin, these changes couldn't have been connected to clinical changes such as local erythema, physiological defence reaction of the skin against this type of aggression (96).

In melanoma, mutations of p53 are considered tardive events which occur in advanced illness cases. In non-melanocitary cancer cases, these mutations have been observed even on premalignant lesions such as actinic keratosis (KA). KA is considered to be a type of cutaneous squamous cell carcinoma in situ. Regarding the percentage of these mutations, there are different opinions of authors. Some of them state that these mutations are found 92-100% in melanoma; others state that the percentage is 7-27%. Mutations of this gene have been found in approximately 66% of cases with KA (97, 98).

There are studies which place the carcinogenic role of the UV radiations as a tardive effect of the tegument. This could explain the lack of connection with local phenomena of reaction type of the skin (table 1.II) (99).

Table 1.II Biological effects of the exposure to UV radiations (99)

<table>
<thead>
<tr>
<th>Acute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythema</td>
<td>Photo ageing (dermatoheliosis)</td>
</tr>
<tr>
<td>Tanning</td>
<td></td>
</tr>
<tr>
<td>Photo alteration of DNA</td>
<td></td>
</tr>
<tr>
<td>Release of pharmacologic mediators</td>
<td></td>
</tr>
<tr>
<td>Local immunosuppression</td>
<td>Cutaneous carcinogenesis</td>
</tr>
<tr>
<td>Epidermal hyperplasia</td>
<td></td>
</tr>
<tr>
<td>Activation of anti-oxidant pathways</td>
<td></td>
</tr>
<tr>
<td>Vitamin D photosynthesis</td>
<td></td>
</tr>
</tbody>
</table>
Alterations of the DNA level can be of two types: genetic translations and mutations. UVA and UVB radiations can determine local immunosuppression by producing less antigen presenting cells or by increasing the production of immunosuppressing cytokines as in the case of UVB radiations. The local immunosuppression is directly proportioned with their dose. Initially, the immunosuppression is caused by UVB and then there comes UVA to maintain this effect and finally, there is a cumulative effect given by the interaction between the two types of radiations. However, UVC radiations do not manage to penetrate the ozone layer because of their short wave length; this is why they do not reach the Earth and they are not involved in the cutaneous pathology (100).

The low occurrence of the cutaneous neoplasia in people with dark skin is, first of all, the result of photo-protection ensured by the big quantity of melanin in the epidermis. This melanin filters the UVB radiations twice more in the dark skinned people than the Caucasian ones. It is estimated that the UVB dose necessary for producing an erythema is approximately 6 to 33 bigger than in people with white skin (101).

From a histological point of view, the photo-lesions at the level of epidermis are characterised by severe atrophy and hyperplasia as well as changes of keratinocytes nuclear atypia type. The hyperpigmentation which appears as a result to UV exposure can also be mediated by cytokines which control the growth, differentiations and synthesis of melanin pigments. Other types of cells suffer changes as well, for example Langerhans cells from the photo-aged skin are in small number and present various morphological changes (102).

At the level of the dermis, after a long exposure to UV, we can notice a decrease in collagen quantity, especially collagen fibres VII as well as the disintegration of elastin fibres. The raise of reactive oxygen species at the level of tissues is generally accepted nowadays as being a direct effect of the radiation with UVA and UVB. The induced lesions and the level of DNA include mono and dicatenary scindation at the basis level and even the formation of abasic sites (103,104).

We also mention the arsenic as an external factor involved in the pathogenesis of cutaneous neoplasia. Agency for Research on Cancer regards the arsenic as being a first class carcinogen because of the risk of cutaneous neoplasm but also the risk of inducing neoplasia at the level of lungs and bladder. The most frequent cause for transmission is drinking contaminated water (105).

Another chemical agent involved in etiopathogenesis of cutaneous neoplasia is psoralen. A long term use of 8-metoxypsoralen in association with ultraviolet type A (PUVA therapy) in the case of patients with psoriasis has been associated with a raise of incidence of squamous cell carcinoma in these subjects (106).

The emitted radiations by using Solar Tanning are considered to be class A carcinogens by World Health Organization in 2009. These UV are associated with an increased risk of developing melanoma but also with a 2 or 3 times raise in the NMSC cases (107).

Taking into consideration the changes which take place in neoplasia and which regards different signalling pathways, the ideal therapy should result from a combination that aims at
different sites of more signalling pathways in order to have satisfactory clinical results (figure 1.4). This thing is supported by the fact that dacarbazine is efficient in only 15-20% of patients with melanoma (108).

P16 gene is another gene of tumour suppression which codes a protein. It is frequently activated in melanocitary tumours as well as non-melanocitary ones. The gene is found on the short arms of chromosome 9 (9p21.3) and plays the role in deceleration of cellular progression from G1 phase to S phase (109).

Alterations of this gene are present in approximately 15% of families with FAMMM syndrome (familial atypical multiple mole melanoma), illness also known under the name of Familial Dysplastic Nevus Syndrome or Atypical Nevus Syndrome (110).

The over expression of p16 protein is considered to be a differentiation criteria between modular melanoma of spitzoid type and Spitz nevus for pediatric population (111).

Other neoplasia mentioned to be related to p16 alterations are: acute lymphoblastic leukemia, biliary tract tumours, mammary and pancreatic neoplasia (112).

Clinical experience and results of experimental studies have demonstrated that cutaneous neoplasia can be successfully treated by surgical excision of lesion only in incipient stages when the evolution prognosis is more improved. On the other hand, when the patient is seen in an advanced stage of the illness, local therapy is already not possible and the attention of the doctor is directed to alternative solutions such as inhibitors of mutant proteins (113).

**Figure 1.4 Major pathways involved in NMSC neoplasia**

CDKN2A= cyclin-dependent kinase inhibitor 2A; GSTT1= glutathione S-transferase Theta-1; CYP2D6=cytochrome P450, family 2, subfamily D, polypeptide 6; PTCH1= patched homolog 1; XPC= xeroderma pigmentosum complementation group C; MC1R= melanocortin 1 receptor; TP53= tumour protein p53 (114).
Basal cell nevus syndrome (Gorlin syndrome), an affection with autosomal dominant transmission is characterized by occurrence of a mutation at PTCH 1 (9q22.3) level. Somatic mutations of PTCH 1 appear in sporadic basal cell carcinoma with a percentage of up to 86% (115).

Mutations of MC1R are associated with a high risk of melanoma but recent data supports the role of MC1R in NMSC etiopathogenesis. The GSTT1 polymorphism is associated with the susceptibility to developing NMSC (116).

P450 cytochrome is the key element of some major pathways such as steroidogenesis or melanin synthesis. Any functional changes caused by genetic or epigenetic factors will lead to changes in internal homeostasis. The CYP2D6 polymorphism is associated with the risk to develop NMSC or other forms of neoplasia (114).

As far as theory of protection of estrogen in NMSC is concerned, this has been tested on laboratory mice only. It has been proved to be valid in the process of animal tumorigenesis. Mancuso et al. has demonstrated this role as a result of research in which appearance of chemically induced CSC has been compared, in different groups of mice. The study concluded an important raise of induced CSC in ovariectomized mice comparing to the ones in which the ovaries had not been removed. Additionally, male mice also presented a raised incidence of neoplasia comparing to the female mice that had undergone the same procedures (117).

1.9 Diagnosis of cutaneous neoplasia

Dermoscopy or microscopy is one of the most modern methods of diagnosis and monitoring of lesions of pigmentary type. The principle of this method consists in magnifying the picture more times with the help of some lens in association with the use of a light system (126).

Videodermoscopy is an advanced system in digital dermoscopy. It analyses pigmentary lesions and allows early diagnosis of skin cancer. It also allows the storage of analysed images with the possibility of monitoring changes in time (figure 1.6; 1.7).
Figure 1.6 Videodermoscopic examination of a pigmentary type - glabrous skin (own collection)

Figure 1.7 Videodermoscopic examination of a pigmentary lesion- hairy skin (own collection)

Cutaneous echography of high frequency is a method which is more frequently used as an additional investigation of non-intrusive type of different tumour cutaneous lesion (figure 1.8).

By using this method we can measure the depth of the skin, by invading the lesion in its depth, the presence of recurrence or therapy effects are monitored (130, 131).
The clinical diagnosis assumes a large experience in the field; the best standard is represented by a histological examination. The anatomopathologist is the one who will always give a certain diagnosis regardless the experience of clinician.

In the case of basal cell carcinoma shave or punch biopsy is preferred. The most used type of biopsy is excisional biopsy in cases of lesions which are suspected of being malignant. Aspiration biopsy with a fine needle is used only in cases of lymphadenopathy, by aspiration from the level of lymphatic node. This way, the level of extension of the melanoma is specified. It is a little intrusive method and with a great value of stadialization.

Surgical excision of lymphatic nodes or some groups of nodes is an intrusive method that requires general anesthesia and it is done if that ganglionic group is close to the tegument. Methods like: thoracic x-ray, computed tomography, nuclear magnetic resonance, positron emission tomography are frequently used to check the changes that occurred at the level of lymphatic ganglions or at the level of various organs that could be affected by melanoma (lungs, brain). In general, blood tests are not used in the case of cutaneous neoplasia. However, we must mention that a rise in lactate dehydrogenase (LDH) in the case of melanoma often means a distant metastasis of this neoplasia, the level of LDH is used as stadialization criterion.

2. PERSONAL PART

The research proposed within this study has had as a main goal the definition of the profile for cutaneous neoplasia in the North-East region of Romania. The objectives have been achieved through two different studies: a retrospective study which evaluated the prevalence and spread of this illness in this region of the country and the second objective which was a study of molecular biology in which I have used Real Time PCR method (RT-PCR) for evaluation of one of the most important factors connected to the neoplastic process, the mutations which occurred at the level of the human DNA.

The objectives of the retrospective study regarding epidemiologic data of cutaneous neoplasia in the North-East part of Romania have been the following:
establishing the occurrence of main forms of cutaneous neoplasia in the North-East part of Romania;

- description of the demographic characteristics (spread according to age, gender, environment) and histopathologic characteristics in order to emphasize those particular aspects, specific to patients from the reference environment;

- correlation of neoplasia occurrence with exposure to certain risk factors, in order to find some adequate prevention measures;

- establishing the evolution of cases over a period of five years;

- determining the impact that the diagnosis of cutaneous neoplasia has on the quality of life;

- establishing the level of efficiency of various therapy measures in the study group.

### 2.1.2 Results

From the total of 1230 patients, 623 have been males (50.6%) and 600 have come from rural environment (48.7%).

Table 2.VI Spread of skin cancer according to gender and residence

<table>
<thead>
<tr>
<th>Type of cutaneous neoplasia</th>
<th>Total</th>
<th>Male</th>
<th>Female</th>
<th>Urban</th>
<th>Rural</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>CBC</td>
<td>675</td>
<td>54.9</td>
<td>335</td>
<td>53.9</td>
<td>340</td>
</tr>
<tr>
<td>CSC</td>
<td>217</td>
<td>17.6</td>
<td>123</td>
<td>19.8</td>
<td>94</td>
</tr>
<tr>
<td>MIXT</td>
<td>26</td>
<td>2.1</td>
<td>12</td>
<td>1.9</td>
<td>14</td>
</tr>
<tr>
<td>MM</td>
<td>119</td>
<td>9.7</td>
<td>64</td>
<td>10.3</td>
<td>55</td>
</tr>
<tr>
<td>Others</td>
<td>193</td>
<td>15.7</td>
<td>88</td>
<td>14.1</td>
<td>105</td>
</tr>
</tbody>
</table>

Kruskal Wallis Test

- Chi-square=6.081; df=4; p=0.193

- Chi-square=43.672; df=4; p=0.001
Basal cell carcinoma has been diagnosed in 675 patients, representing 54.9% from the cases, with a nodular form being the most frequent (40.4%), followed by the one with adnexal differentiation (21.1%), keratotic (14.8%), superficial (6.8%) and pigmented form with 6.1% (table 2.VII).

All forms have been represented by similar percentages to those from specialised literature and as far as the localisation is concerned 81.6% have been found at facial level, 11.4% at the level of the body and only 11.1% at the level of the scalp. Data support once again the theory according to which UV rays play an important role in the etiologic process of neoplasia outbreak.

Squamous cellular carcinoma has been diagnosed in 217 patients representing 17.6% from the total of neoplasia cases. The most frequent histological form was the keratotic one, 81.9%, and melanoma was diagnosed in 9.7% cases, with an Clark III invasion level as being dominant (33.6%) (Table 2.VIII, table 2.IX). We also mention that there have been a reduced percentage of anatomopathological bulletins in which the histological form of neoplasia is not specified.

Table 2.XI The histopathological pattern of melanoma

<table>
<thead>
<tr>
<th>Histologic Pattern of melanoma</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clark I</td>
<td>2</td>
<td>1.7</td>
</tr>
<tr>
<td>Clark II</td>
<td>4</td>
<td>3.4</td>
</tr>
<tr>
<td>Clark III</td>
<td>40</td>
<td>33.6</td>
</tr>
<tr>
<td>Clark IV</td>
<td>34</td>
<td>28.6</td>
</tr>
<tr>
<td>Clark V</td>
<td>13</td>
<td>10.9</td>
</tr>
<tr>
<td>MTS de MM</td>
<td>18</td>
<td>15.1</td>
</tr>
<tr>
<td>Recidive</td>
<td>2</td>
<td>1.7</td>
</tr>
</tbody>
</table>

The frequency of melanoma was higher in the rural environment than the urban one (82% versus 68%), but the melanoma was found more among patients from urban environment (12, 3% versus 5, 9) (p=0.001).
2.1.3 Discussions

The data obtained due to this study was appropriate with the specialized literature as far as the percentage of types of neoplasia is concerned, localization at the level of body, percentage according to gender and environment (180).

Because this study was a retrospective one the variables introduced in it were found on the observation sheets of patients. We had to give up on information regarding the occupation of the patient, clinical presentation of the tumour (dimensions), risk factors (exposure to UV radiation, smoking, immunosuppression) because not all the sheets contained these indicators. This way we mentioned some limitations of this study:

- lack of information regarding the dimension of the squamous cellular carcinoma (maximum horizontal diameter) is considered a factor of negative prognostic. At the same time, this diameter is cited to be bigger among the neoplasia lesions located at the level of limbs (181, 182).
- the connection between the body mass indicator and the frequency of melanoma. In the specialized literature it is cited an association between overweight people, obesity and the risk of developing cutaneous melanoma. From the data we obtained we could not do such an association (183).
- we did not succeed in pointing out a significant rise from a statistical point of view of the number of cutaneous neoplasia cases. One cause could be the fact that the study was done over a period of only 5 years;
- the study did not include pediatric patients as well because Saint Spiridon county hospital serves only adult patients. However, the risk factors for developing cutaneous neoplasia are the same as for adults, just mentioning that ABC rule is many time impossible to apply in the case of children and the predominant form of melanoma is achromic (184,185).

In the case of basal cell carcinoma, the highest frequency was at facial level. The second place is occupied by the body area and then the limbs. Our data corresponds from this point of view with other studies which place this clinic entity at the level of cephalic extremity. This confirms once again the primordial role that the exposure to UV radiation has in the etiopathogenesis of the illness (186).

The nodular form was the most frequent in our group of patients. This situation is explained by the fact that at the level of the cephalic extremity we frequently find this histological entity, followed by the superficial form of basal cell carcinoma, more frequently met in lesion located on the body, in male patients (187).

We could not find a high frequency of basal cell carcinoma in male patients, but the incidence was the highest in the 6th decade of life as also mentioned by other data from other studies (188).

If specialised studies support the idea that this type of neoplasia develops more frequently in the elderly and only exceptionally in young people (when we associate pathologies
with albinism, xeroderma pigmentosum and Gorlin Syndrome), our results are in accordance with this data as well. The basal cell carcinoma is most frequently met in 50-59 years age group (189, 190).

As far as the second type of neoplasia according to frequency- squamous cell carcinoma, we succeeded in concluding the following:

- its frequency corresponds to the one mentioned in the specialized studies, most authors recognise this type of neoplasia being the second as frequency between NMSC, after the baso cell carcinoma (191, 192).
- the most frequent age group for squamous cellular carcinoma was ≥ 90 years old.
- as localization, it is considered to appear more frequently in photo exposed areas and affects male patients more. Our data have supported both characteristics. 76% of lesions appeared at the level of the face and the male patients have has a higher percentage 56, 68 (193).

As far as the melanoma is concerned:

- we confirmed the higher frequency among male patients, the double percentage in the urban environment against the rural areas (12, 3 versus 5, 9) as well as predilect location at the body level (44, 5%).
- the age of the occurrence of this neoplasia was unfortunately a lower one comparing to other neoplasia cases, most frequently this melanoma being presented by patient of 20-29 years of age.

Location at the level of the body is more frequent among the specialised studies, no matter the age and gender of the patient, out result corresponding to international data. More recent data support the fact that, the frequent location at the level of the body could be more obviously associated with an intermittent exposure to UV rays and use of solars (194).

The major age group for melanoma in this study has been lower comparing to other results. It is estimated that the average diagnosis of melanoma could be around the age of 52, and lower for the Caucasian race (10).

Our results have shown differences between the two environments discussed according to the two big categories of cutaneous neoplasia: NMSC more frequent among rural patients, while the melanoma was more frequent among the urban patients. This difference between the patients backgrounds are reported throughout Europe as well, related to certain illnesses or various population subgroups.

Generally, these differences could be explained by using more variables:

- outdoor activities (a lot of our subjects were former farmers), activities which mean chronic exposure to UV. This aspect could support the high frequency of NMSC lesions in the rural areas.
- educational differences which create impediments for the patient to contact a specialist

From this point of view, the deficit is most frequent in the rural areas, environment in which it takes a lot of time to reach medical help for a health problem and the fatalism that comes together with the idea of neoplasia is still very much frequent.
accessibility to a health service. If in the urban area, a patient has a multitude of options regarding health centres, in the rural area this impediment leads to sub-diagnosis, including skin cancer, idea that could support the big number of melanoma in the urban areas.

- accessibility to public information. It has been noticed that a lot of patients diagnosed with melanoma have asked for a first specialised check as part of the various information campaigns led at local and national level. These programmes illustrate the risk of developing a certain neoplasia and this way the patient is aware of them and does something about them where necessary. The number of these campaigns is very small in the urban area and passing on information is also difficult (for example, lack of access to internet) (195, 196, 197, 198).

The fact that the most frequent cases of neoplasia are met in the elderly makes the diagnosis more difficult to establish, the prognostic to be worse, treatment to be chosen according to comorbidities and the therapeutic response to depend on other associated therapies. Comparing to the patients diagnosed with melanoma, for patients with NMSC, one of the most important factors of prognostic is represented by associated comorbidities and decrease of functional resources of the body (199, 200).

Data from specialized literature offer as a risk factor for recurrence the safety of oncologic margins from surgical excision. Data obtained by us show a quite high degree of lack of specifications regarding this aspect: 5, 6% of basal cell carcinoma, 7, 4% of squamous cell carcinoma and 24, 4% of melanoma did not have a specified safety limit. In conclusion we cannot calculate a recurrence risk at these patients from the point of view of this aspect (201).

We did not have patients under the age of 20 throughout our study and we were not able to calculate the frequency of cutaneous neoplasia occurrence (malignant melanoma) in pediatric population. Data show that only 1-4% of melanoma occur under the age of 20. The risk factors are similar as for adult patients and the histological characteristics are also similar to the ones described for other age groups (202).

Despite the majority of studies supporting the raise of incidence of cutaneous neoplasia at a global level, there are contradictory opinions as well, which support the fact that this raise is not real but only a consequence of the fact that there has been an increase in diagnosis methods. Another reason is that some classifications have been modified (the inclusion of tumours of borderline type in the diagnosis of cutaneous neoplasia) and screening methods have been improved or various entities which have been considered benign have been included in the category of neoplasia in situ.

This last idea would support our result and would explain why the increase of incidence is not correlated with a raise of mortality, the mortality due to cutaneous cancer remaining at the same levels in the recent years (203, 204, 205, and 206).

A statement that would support the raise of incidence would be the fact that, the increasing number of cases has proved that it had not been influenced by socio-economic factors. In other words, no matter the accessibility to screening method or to a diagnosis procedure, the incidence is not modified (207, 208).
2.2 STUDY REGARDING BRAFV600E MUTATION INCIDENCE AMONG THE CUTANEOUS MELANOMA CASES

Data of the retrospective clinical study have been completed by this prospective study, done within the Microbiology Laboratory of University of Medicine and Pharmacy Iasi. Real Time PCR method was used to investigate the presence of BRAF V600E mutant gene at the level of cutaneous melanoma.

What does BRAF V600E represent? BRAF represent a serine/threonine protein kinase involved on MEK/ERK pathway. Mutations of this protein are most frequent in melanoma: BRAFV600E (approximately 80%) and BRAFV600K (5-30%) (210).

Why did I choose the melanoma?

Because this type of neoplasia, even if it represents only 4% of all clinical forms of skin cancer, is responsible for almost 65% of deaths cause by cutaneous neoplasia (211).

Because access to therapy with Vemurafenibum is possible only after the patient is proven to have this genic mutation, patients who do not have this mutation are not eligible for this treatment.

Because not all those diagnosed with advanced melanoma can afford this test, as it requires two different methods of molecular biology, DNA extraction and then the identification of mutation, these methods are not accessible to any laboratory and the costs involved are an impediment when testing only one subject is requested.

The objectives of this study have been:

- the main objective- improving the diagnosis algorithm for cases of cutaneous melanoma in order to improve the prognosis of patients;
- secondary objectives:
  - investigation of molecular objective of patients with cutaneous melanoma from a target group of study;
  - determination of frequency for BRAFV600E mutation on this group of patients;
  - establishing some correlations between the presence of mutation and the different clinicopathological characteristics: age, gender, location at cutaneous level, histological form, TNM stadialization, Level of Clark invasion, Breslow width;
  - clarification of some terms and techniques of molecular biology used in paraclinical diagnosis of cutaneous neoplasia;
  - establishing the efficiency of diagnosis kits, used to improve the algorithm of paraclinical diagnosis;
  - identification of some factors which generate possible error of using the method.
2.2.2 MATERIAL AND METHOD

I have organised a study group out of 30 patients who had been diagnosed with cutaneous melanoma. The excised parts have been archived using FFPE, from which I extracted the DNA in order to test it for BRAF V600E mutation identification. The used method was Real Time PCR, which supposes a genic amplification, frequently named PCR (Polymerase Chain Reaction), or molecular photocopying. This represents the reaction through which the fast generation of multiple copies of some target nucleotide sequences DNA and RNA, detectable copies through corresponding probe. The amplification is done with the help of thermal cycler and the mic of reactions contains the following:

- the DNA obtained through sampling after extraction
- TAq DNA polymerase (an enzyme obtained from Thermophilus aquaticus which facilitates the synthesis of complementary chain of nucleic acid)
- enzymatic co-factors (Mg2+ and/or Mn2+)
- primers
- deoxy nucleotides
- Amp Erase (enzyme that selectively amplifies the target and destroys the contamination products).

PCR takes place in three steps:

- denaturation- the DNA is separated (denatured) in two catena after heating the reaction mixture at 94° C
- hybridization of primers- binding primers to 3’ endings of the two catenae as a result of lowering the temperature to 55-70°C
- Elongation step- DNA polymerases expands the bound primers along the target matrix and will produce a DNA amplicon

The described steps do not happen without stopping. After a certain number of cycles (on average 30-40), the amplified sequence does no longer accumulate exponentially, the reaction enters in a linear phase also called ‘the plateau phase’. The whole reaction is done automatically and takes a few hours.

As part of the PCR conventional technique, the amplified product is detected in the final stage of the analysis on an agarose gel, after the amplification has ended and when different inhibitors of reaction may appear. Comparing to PCR, Real Time PCR technique allow that the detection of the product take place in real time (the quantification of target is done before the appearance of inhibitors) (figure 2.2.4). Real time detection of products obtained from PCR has become possible by including as part of the reaction a fluorescent molecule which allows the detection of DNA quantity increase, proportional raise with the fluorescent signal, this way fluorescence can be measured within each amplification cycle (141).
Other advantages of the Real Time PCR technique versus PCR conventional reaction are:

- the preparation of the gel is no longer necessary
- it requires less time
- it is an easier process

Another characteristic of Real Time PCR reaction is represented by the amplification curves which increase after a number of cycles. The number is proportional with the initial DNA matrix (template) quantity and comparisons to the values of the curve will bring data regarding the quantification of the desired product (212).

In Play Set up section, the wells as being standards, samples to test (unknown), positive check-ups, negative check-ups as well as wave length at which amplification will be interpreted. In Thermal Profile Set up, the number of cycles and temperature according to kit producers’ specifications are decided.

A preliminary analysis of the obtained results by using Real Time PCR reaction involves checking the following elements: examination of amplification curves, checking the positive controls and negative controls, 'threshold' adjustment, adjustment of background line, specific analysis of the event.

$C_t$ parameter (Thresholds cycle) represents the fractional number of the cycle which coincides with increased fluorescence on the baseline, meaning the detection of the product through PCR. From this moment the exponential phase of the reaction amplification actually begins. A value of $C_t$ of 40 or bigger indicates the fact that no amplification has been done and the obtained value cannot be taken into consideration.

The first Real Time PCR instrument was ABI7700- fiberoptical/ laser, used for the first time in 1996. After this one, there have been the following: ABI 5700-CCD, Idaho Technology LightCycler- capillary tubes, Eppendorf Realplex, Rotor-Gene from Corbett Research, iCycler de la Bio Rad, Strata gene Mx3005p, BioRad CFX96, Applied Biosystems 7500 Fast, Roche LightCycler 480, Fluidigm Biomark (212).

Among the most used Real Time PCR instruments nowadays, we mention the ones produced by ABI Company (Applied Biosystems) and that is ABI7500 and those of Roche - Roche Applied Science (LC480).

In practice, the most applications of the Real Time PCR method are represented by:

- detection/quantification of pathogens from the human, animal or food probes
- description of chromosomal aberrations
- the study of neoplasia (detection of sequence and tissue in which certain oncogenes are expressed in various malignant processes
- forensic medicine (molecular analysis of the different biological probes)
• analysis of genes expression
• protein expression
• viral quantification
• anthropology studies
• taxonomy molecular, systematic and phylogeny studies
• analysis of the cellular immune response in peripheral blood
• analysis of nucleotide polymorphism (SNP = single nucleotide polymorphism).

The sensitivity and specificity of PCR methods depend on more factors: the used primers, the dimension of the amplicon, efficiency of DNA polymerase, conditions of reaction to happen, etc.

Usually, DNA extraction from the paraffin embedded probes is done in three steps: deparaffinization, digestion and purification. At the present there is a wide variety of DNA extraction kits on the market. Each producer tries to optimise the method, to reduce the work time and this way making it accessible to a large number of users (213). For instance, on the Romanian market there are DNA extraction kits from FFPE and it does not require deparaffinization. In our case, the SNP percentage from probes is calculated based on delta Cq method. The proportion of SNP in the tested probe is then corrected by comparing it to a standard in which SNP is present 1%.

Calculation model

Interpretation is done in three steps. First Cq delta values are used to calculate relatively the levels of detection between the wild type and mutant sequence, for biological probes as well as for 1% control probe. The obtained value is then converted into a percentage. This 1% from the probe value is used for normalization of the signal for the biological probes by creating a K value which corrects any variation within the probes and any setting up of the qPCR instrument.

Used equations:
Delta Cq = $2^\Delta$ (BRAF Wild type - BRAF V600E)
Conversion percentage = $\frac{1}{\Delta \text{Cq}} \times 100.$

Necessary steps:

1. Calculation of percentages from the biological probes and positive control
Example:
Teste biological probe (figure 2.25)
BRAF Wild type Cq = 26
BRAF V600E Cq = 32
Delta Cq = $2^\Delta$ (26-32) = 64
Conversion percentage = ($1/64$) $\times 100 = 1.56\%$
Calculation of positive witness (figure 2.26)

BRAF Wild type $C_q = 24$
BRAF V600E $C_q = 32$
Delta $C_q = 2^\Delta - (24-32) = 256$
Conversion percentage = $(1/256) * 100 = 0.39%$

In an ideal situation, this 1% from the standard of positive control should lead to a 1% result when $C_q$ delta method is used. Anyway, because of all the variations that appear in experimental conditions but also because of different hardware platforms, the calculated value
can be different. The 1% positive control is used as a normalization factor to correct any systematic error that occurred during the experiment.

Normalization factor (K) = \frac{1\% \text{ proportion} 1\% \text{ positive}}{0.39\%} = 2.56

2. Correction of tested probe using the normalization factor

Mutant Percentage of Probe = \text{measure percentage} \times K

Percentage of teste probe = 1.56\% \times 2.56 = 4.0\%

Interpretation = '4\% from the tested DNA probe presents mutations in proportion of 94\%, comparing to wild type'

The detection limit of this kit is 0.1%. Results which mention detection at lower levels 0.1\% should be considered negative.

**DNA EXTRACTION**

At the present there is a varied offer of commercial kits for DNA extraction paraffin embedded probes. According to their availability in Romania, we chose to purchase the following kits: PureLink® Genomic DNA Kits / Life Science (Invitrogen), ReliaPrep™ FFPE gDNA Miniprep System (Promega) and Innu PREP FFPE DNA KIT (Analitik Jena). The work protocols for the above mentioned kits recommended the use of the same amount of tumour tissue: PureLink kit- 1-8 sections de 5-15µm, ReliaPrep kit- sections between 5-50 µm and Innu PREP FFPE DNA C16 kit- sections of 10µm.

Figure 2.49 The principle of the semi-automatic extraction (Black PREP FFPE DNA KIT/Analytik Jena).
In order to check the efficiency of the used kits we used the quantity of DNA and its purity as values which have been measured with the help of the NanoDrop.

**IDENTIFICATION OF BRAFV600E MUTATION**

As far as the principles of the QUASA testing are concerned, we mention the following particularities:

- quasa (quantitative allele specific amplification) is an innovative method of detection of some mutations from different probes
- quasa is a very sensitive method which allows the detection of mutations that are found in minimum quantities in the tissue
- quasa is based on a method that belongs to Primerdesign which uses modified primers, the conditions of the modified cycles and a modified master mix (figure 2.50)
- as part of this method, the 3'terminal end is made so that it could bind to the mutant base.

In the probes in which mutation is present, an efficient amplification will be represented by (BRAF V600E) mutation detection and blocking of the wild type (Figure 2.51)

The work model of the kit that is used is a specific one which allows the analysis of maximum 5 probes.

![Figure 2.51 Braf thermal profile/ quasa](image)

23
The thermal profile of the experiment, specific to those from Primerdesign presented 1 cycle from 25°C to 95°C and 55 cycles made of 2 segments distributed in the following way: 10 seconds at 95°C, 3 seconds at 50°C and 15 seconds at 72°C, but the second segment with 10 seconds at 95°C, 30 seconds at 60°C and another 15 seconds at 72°C.

![Figure 2.52 Modified polymers](image)

### 2.2.3 RESULTS

**The results obtained with Purelink® genomic DNA kits / Life Science from Invitrogen**

Among the probes purified with Purelink® we obtained DNA values between 'too low' for 4 of probes and 12, 485 ng/µl. The majority of the probes, 8 of them, had less than 1 ng/µl.

We used 5 probes, those which have bigger DNA quantities to go further and do the detection of mutation. Among those 5 probes, only two have amplified for BRAFV600E and none for BRAF Wild Type. Positive controls have been present and correct.

In conclusion, DNA quantities purified by using the first kit have been very small, insufficient for a further reaction of amplification.

**Results obtained using RELIAPREP™ FFPE gDNA MINIPREP SYSTEM kit from PROMEGA**

In order to minimalize the risk of running out of probes without an optimum result, I have extracted the DNA from only two probes which contained a big quantity DNA (probe 1 = 17, 9 ng/µl and probe 2 = 181 ng/µl). The first probe has amplified for BRAFV600E, and the second one has amplified only for BRAF Wild Type. The positive controls have been present and correctly indicating the fact that the procedure was correct.

Results:

Probe 1- A260/280 = 2,571
Probe 2- A260/280 = 2,034.
The results obtained by using Black REP FFPE DNA KIT from ANALITIK JENA

From 16 tested probes, 9 have amplified for BRAFV600E as well as BRAF wild type. In the following part we tested another 14 probes from which 9 have amplified for BRAF V600E. Final result- from the 30 amplified probes, 18 have been positive for BRAF V600E (60%). Positive controls have been present and correct indicating the fact that reaction was correctly done.

<table>
<thead>
<tr>
<th>Well</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.58 Ct values for the tested probes

In A-C wells we tested for BRAF V600E, in F-H wells we tested for BRAF Wild Type. The results -9 probes have amplified for BRAF V600E as well as BRAF Wild Type.
2.2.4 DISCUSSIONS

Recent data show that melanoma is in fact a neoplasia with distinct molecular characteristics whose understanding represents a change to a better therapeutic option. The present study has aimed to define the molecular profile of the cutaneous melanoma and comparison of the obtained results to the already existing ones. At the moment, molecular studies are the ones which bring forward new therapeutic options in advanced stages of the illness and which try to implement a new concept of the medicine and that is personalised medicine.

The access to FFPE represent more benefits: the possibility to process the tumoral tissue at any post moment- excisional without using an invasive act for the patient, stability of material for a long period of time (ten years), easy manipulation of probes.

The Real Time PCR technique represents one of the easiest techniques from molecular biology which can be applied on this type of tissue in order to detect the genic change that was looked for.

The study group was formed by 30 patients, the majority coming from the rural area (56% vs 43%). 43% of these 30 patients had another neoplasia before and the gender percentage was similar. We observed a majority of patients over 60 years of age. The histology of the malignant melanoma has shown a percentage of 43, 3% epithelioid melanoma followed by the nodular form with a percent of 43, 3%.
For the detection of BRAFV600E mutation we have use the method of those from Primerdesign UK-quantitative allele specific amplification (quasa) kit. Quasa is a sensitive method for the detection of mutation even if this is present in low quantities in the probe of tumoral tissue (up to 0, 01%) a probable thing for probe embedding (217). Quasa experiments have been validated by positive and negative. In the case of used probes, 18 of 30 have been positive for BRAFV600E mutation. The Values of Cts from the tested probes have varied between 27, 82 - 27, 82, and those of positive controls between 21, 25 - 29, 22.

In the last decades, the detection of some antigens associated with skin cancer have had varied results, but the immunotherapeutic protocols are being defined at the present. For example, vemurafenibum, discovered in 2002, licensed in 2011 by FDA (Food and Drug Administration) under the name of Zelboraf® is a model of therapy obtained by a genetic study of mutations which appeared within the process of cutaneous neoplasia (218).

Vemurafenibum is an inhibitor of kinases with oral administration which block the mutant form of BRAF protein. This medicine has been approved as a therapeutic option for cutaneous melanoma which is unresectable or already in metastasis for which classical or IL-2 chemotherapy was applied before (219).

BRAF gene is a gene located at the level of the long arm of chromozome 7, position 34 and it is formed by 18 exons. BRAF gene codes a protein which belongs to raf family raf serine threonine kinase. B-raf protein plays an important role in regulation of MAP-kinases (mitogen activated protein kinase) in RAS/MAPK transmission pathway involved in the cellular growth and splitting, differentiation, secretion and apoptosis (220).

The activation of a series of changes in the phenotype of a cell happens in more steps through which the signal follows cascade of kinases with a role in activation of various proteins. The Kinases are enzymes which are involved in the transmission of various cellular signals with a normal function of the cell such as growth and division/splitting. BRAF if the most frequently mutant protein-kinases present in cancer. It is estimated that approximately 8% from solid tumours contain mutations of BRAF V600 type and approximately 40-60% of the cutaneous melanoma present mutations at this level, mutations which lead to activation of signalling pathway of MAPK. Normally, this signalling pathway is responsible for the transmission of extracellular signal through the membrane towards the nucleus. In melanoma, and not only here, instability of this pathway appear as a result of genetic mutations at the level of B-RAF and RAS genes (221).

Approximately 80-90% of these mutations result in substitution of valine with glutamic acid at position 600 (V600E) (222). BRAF 600K mutation appears in 20% of the melanoma cases and more frequently in melanoma in situ or in the cases of lentigo maligna.

BRAF V600E mutation is also found in neoplasia such as: histiocytoma with Langerhans cells, colon cancer, thyroid papillary carcinoma, astrocytoma or leukemia with hairy cells (223,224).

BRAF mutation of V600E type lead to hyper activation of MAPK pathway which in turn modify the rhythm of cellular division/splitting, induce the proliferation of neoformation blood
vessels by promoting EFG (endothelial growth factor) or overexpression of proinflammatory cytokines such as IL-8 (225, 226).

DNA extraction from paraffin embedded probes has the big advantage of being available to any researcher. On this type of tissue there is based most of the retrospective epidemiologic studies which have as a topic genetic mutations (227).

The quality and quantity of the extracted DNA is an important step in the molecular testing, and this thing is influenced by a great number of factors: human factors (selection of appropriate tumoral block, marking the area with the biggest tumoral content or quality of microdissection which need to contain a maximum quantity of tumoral cells, contamination during manipulation) and factors that are strictly related to the processed tissue (the period of paraffin embedding or rehydration within the deparaffinization step)(228).

Paraffin embedding also favours the production of DNA links and various proteins, link which negatively influences the extraction of a quality DNA or even fragmentation of DNA (229, 230).

In our case, the chosen probes were from 2013-2014 in order to have small period of paraffin embedding, the blocks have been selected by an anatomopathologist with experience in the field of oncology and the sections have been done after marking the interest area beforehand. In order to avoid contamination, between each probe there has been used a solution for DNA removal.

The choice of this method for the detection of mutation has been made according to the specialised present studies. Real Time PCR is mentioned among the recommendations of experts in the field (231).

As far as the principle of DNA extraction is concerned, this can influence the result of PCR reaction. We identified as being available approximately 69 kits, only 35 are specific to DNA extraction, 22 are used for ARN extraction and 12 of which extract DNA and ARN. FFPE deparaffinization with xylene is one of the most frequently used methods. However, in order to eliminate these easily inflammable compounds from the steps of PCR reaction, more and more producers have created less toxic chemical compounds, which eliminate paraffin more rapidly and efficiently. There have been taken further steps and some producers have eliminated paraffin completely as a step in DNA extraction. This way, this step has been omitted in the most recent protocols and the obtained data support the fact that the quantity and quality of the extracted DNA have not been influenced.

In the present study, three different kits have been used, having their own extraction principle. By using the first kit, PureLink® Genomic DNA Kits / Life Science (Invitrogen), we have done deparaffinization base on xylene and needed 5 hours of work. Unfortunately, the quantity of the DNA we obtained was not sufficient to continue the protocol of work. The second kit we used, ReliaPrep™ FFPE gDNA Miniprep System (Promega), was based on the usage of mineral oil in the FFPE deparaffinization process. This oil should not be removed and the necessary time for the DNA extraction was shorter (3 hours). As in the case of the second kit, the DNA quantity was not sufficient to continue the reaction. The third kit we used, Innu PREP
FFPE DNA Kit / Analytik Jena, did not suppose we did deparaffinization any longer, and DNA extraction was done semi-automatically with reactives from slides which are pre-charged besides the magnetic beads with all the necessary components for the extraction, from binding, washing until the elution of nucleic acids. At the same time, this kit has a reduced work time of 2, 5 hours. We need to mention the fact that the quantity and the quality of the extracted DNA have been measure with the help of Nanodrop Nano Photometer TM and the result we obtained represented a way of evaluation of work efficiency. The quantity of the extracted DNA was expressed in ng/µl and the quality was expressed by measuring the purity based on spectro-photometry, as well as by calculating the A260/280 rapport. For those probes for which the calculated quantity was 'too low', the DNA extraction was repeated with the help of another kit. This way, the extraction kits have been chose so that we extracted an appropriate quantity tumoral DNA and a superior quality of it. For example, the ReliaPrep FFPE gDNA Miniprep System (Promega) kit, which is mentioned as being one of the best choices for the extraction of DNA from paraffin, embedded probes (232).

By using quasa method we finally managed to detect the presence of mutation in minimum quantities of tissue. Our results emphasize the fact that deparaffinization does not represent a compulsory condition in the extraction of a quality genetic material and this fact is supported by more and more authors (233,234).

During the processing of the tissue (division of the paraffin block, deparaffinization, Real Time PCR analysis) the contamination of DNA can appear, contamination that modifies the final results. In order to avoid this thing, DNA Away solution was used in order to wipe the slide of the microtome between each of the paraffin embedded blocks (235).

Results have shown a frequency of mutation of 60% in case of the 30 patients included in the study, frequency found in concordance with the speciality literature which referred to Caucasian patients. The offered percentage are given by more variables: the sensitivity of methods of detection of mutation, the histological form of the melanoma, TNM stage, the studied population even with the percentage of exposure to UV radiations of the lesion tegument (236).

Our result is also supported by the fact that the study group has included only primary lesions. The fact that BRAF positive melanoma loses this mutation at the level of the metastatic lesions is already known (237).
3.1 FINAL CONCLUSIONS

The proposed research subject is included in the present tendencies of the fundamental scientific research applied in Europe and the world; these pieces of research aim to define a general profile of this type of neoplasia which is more and more noticed. We started from the characteristics of the group of patients and we reached studies of molecular biology - through Real Time PCR reaction respectively. The understanding and use of these methods regarding different forms of neoplasia tropism such as hematodermic neoplasm CD4+/CD56+ positive, as well as improvements of the therapeutic act by selecting eligible patients for inhibitory therapies of the various genic mutations.

The originality of the piece of research along with the multidisciplinary character (dermatology-microbiology/ molecular biology) which imposed the use of Real Time PCR method with the aim of detection of BRAF V600E mutation in cutaneous melanoma probes, consisted as well in detection, for the first time in the North-East region of Romania, of this mutation with the definition of cutaneous neoplasia's profile. From 2008 to 2012, 1230 patients have been selected from whom we analysed 1319 pieces.

It is worth noticing that this study was done in the context that there was no data base which could cover the total number of cases of cutaneous neoplasia discovered in the country.

In order to fill in the obtained data, although it has been done on an important study group of patients, in the future we find it necessary to do studies that could help to complete the present data base for the creation of a national register which could include the main clinical and histopathologic characteristics of the cutaneous neoplasia of the patients coming from all over Romania. The presented data represent the first attempt of the university centre in Iași of this kind. The cases included in this study came from the surrounding counties of Iasi as well (Botoșani, Vaslui, Neamț, Suceava), so representing an overall image of the cutaneous neoplasia in the whole region of North-East Romania.

The statements that support the second part of the PhD research paper are: the raise of the numbers of melanoma at a global level, the low level of the age for appearance of this type of neoplasia, as well as its aggressiveness. All these aspects justify the necessity of testing patients by using different methods of molecular biology in order to start inhibitory therapies where other alternative therapies do not work.

For the detection of BRAFV600E mutation, we used the method proposed by producers who launched Primer Design UK-quantitative allele specific amplification (quasa) kit on the market. Quasa is a sensitive method of mutation detection even if this is very little present in the tumour tissue probe (up to 0, 1%). Quasa experiments have been validated through positive and negative controls. In the case of the used probes, 18 of 30 have been positive for BRAFV600E mutation.
3.2 PERSPECTIVES OPENED UP BY THIS PAPER

Taking into consideration the global tendencies, it is necessary to continue the research focused on the genic expression of cutaneous neoplasia as well as correlating them to clinical aspects of the illness in order to define an individualised therapeutic option which could lead to better survival chances (264).

The Study aimed to set up new correlations between the data from foreign speciality literature and results obtained on study groups in our area, identify predictive paraclinical biomarkers in the evolution of cutaneous cancer, in order to implement in practice of a detailed paraclinical diagnosis algorithm. All these ideas support, in the end, the need of a revision of the present classifications of the cutaneous neoplasia which should include molecular biology criteria as well, besides the ones related to morphopathology.

The necessity of some research in this field is justified by more factors: if we strictly refer to non-melanocitary cutaneous cancer, a pathology which is more frequently present in older population, a tendency of raise in life expectancy in the more developed countries is present, as well as in Romania. If we refer to MM, the age of its appearance/debut is more and more reduced and the aggressiveness that it shows is bigger and bigger. Additionally, the immunocompromised patients are in larger number in the recent years, fact that contributes to a bigger recurrence of the malignant cutaneous cases registered in the last decades (265, 266).

Research from the field of oncology is more and more focused on the importance of the new molecular markers which are more and more part of already present histopathological classifications of the cutaneous neoplasia and which will change the approach towards the neoplasia patient (267).

The detection of BRAFV600E mutation in patients with advanced cutaneous melanoma represents the transition from research to implementation in current practices of a diagnosis algorithm which could classify patients in eligible or not, for one of the first personalised therapeutic in the case of type of neoplasm, respectively Vemurafenibum. This type of medicine is one of those aimed to announce future major changes as far as the management of melanoma is concerned. New therapeutic options are defined for the advanced cases of this illness.

Our results have showed that a quite high percent of BRAFV600E mutation, 60%. This shows that the number of eligible patients for this therapy is quite a large one and the implementation of a diagnosis algorithm which included the detection of genic changes, is a necessity with a practical end. Because the cost represents another impediment in choosing this therapy clinical trials which include patient with advanced melanoma could represent the best option for these cases. These studies could improve the accessibility of patient to molecular therapy, allowing a surplus of information for physicians working in the field.

Taking into consideration the changes which appear in different neoplasia and which are focused on different signalling pathways, the ideal therapy should be thought as a combination which aims at different sites in order to have satisfactory clinical results. Last but not least, for
cases that are resistant to this type of therapy, the association with an immunomodulator could lead to superior results.

SELECTIVE BIBLIOGRAPHY


Thomas NE, Kanetsky PA, Begg CB et al., Melanoma molecular subtypes: unifying and paradoxical results, J Invest Dermatol 2010; 130: 12–14.


