Application of Biomarkers at Different Levels of Breast Cancer Prevention

Doctoral (Ph.D.) thesis

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I.1. Epidemiology and molecular epidemiology of breast cancer

In Hungary 14956 women died of cancer in 2005. In 15.3% of the cases breast cancer could be identified as the cause of death. In our country, in the past 50 years, until 2001, breast cancer mortality occupied the leading position among the causes of death due to tumours developing in females.

The so-called hereditary breast cancers account for 5-9% of breast cancer diseases. The majority of breast cancers are sporadic cases, in which familiar accumulation cannot be observed, they are usually manifested in the elderly ages and they are often agressive. Both external and internal factors play a role in the development of sporadic cancers.

The role of genetic factors in the development of sporadic breast cancers

Besides external environmental risk factors several low-penetration genetic factors are also involved in the the development of sporadic breast cancers. Although these factors alone do not indicate high risk the development of several high-risk alleles should deserve remarkable attention because in this case the chance of developing breast cancer becomes higher.

Those gene polymorphisms belong to this cathegory, which increase individual susceptibility only in a little extent whereas, due to their frequency rate, they influence the additional risk factors of the entire population in a greater extent than rare, high-penetration diathetic alleles do. The chance of developing breast cancer is greater in individuals with several high-risk alleles when they are exposed to environmental carcinogenes.

Several polymorphisms of enzymes metabolizing environmental carcinogenes appear as a risk factor in the human carcinogenesis including the development of breast cancer. Furthermore, the genes of enzymes involved in hormone metabolism and the steroid receptors, as the mediators of hormone effects, also play a crucial role in determining individual susceptibility so they attract the focus of investigations.

The polymorhisms of genes involved in the regulation of the cell cycle, the survival and apoptosis of cells also occur as risk factors in the carcinogenesis. The *Arg/Pro* polymorphism of the p53 protein having a tumour supressive effect belong to this group, as well as vitamine D and its analogues, and also the polimorphisms of vitamine D receptor (VDR) genes mediating its anti-proliferative and apoptotic effect.

The role of vitamie D in the development of breast cancer:

The 1.25-dihydroxy-D_3 vitamine, the active form of vitamine D, play a cental role in the calcium maintenace, in the metabolism of ossification as well as it has an effect on the process of proliferation, differentiation and programmed cell necrosis both in the normal and transformed cells. Low vitamine D level is also associated with the deficiency of oestrogen accompanying the elderly ages so it is related with two important risk factors of breast cancer. This can be explained by the fact that the cholecalciferol synthesis in the skin decreases in the elderly ages and the deficiency of oestrogen reduces the metabolic activation of vitamine D as well as the expression of vitamine D receptor. Since the risk of the development of vitamine D deficiency is much higher in women in the post-menopausal period, who are the most susceptible target group to breast cancer than in younger women the clarification of the exact role of vitamine D in the development of breast cancer can extremly be important.

Since vitamine D elicits its effect through its receptor the function of vitamine D receptor can also be a crucial factor in the individual suseptibility for breast cancer.

The role of vitamine D receptor (VDR) in the development of breast cancer:

Vitamine D receptors are located in the main cell types of the mammary tissue (basal and luminal epithelial tissues, cap tissues, stroma tissues) but their expression is changing in different cell types as well as in time. The dynamic regulation of VDR expression indicates the functional role of $1.25(OH)_2D_3$ in the development of the breast. In addition the VDR gene is a polymorph, several kinds of human vitamine D receptor alleles are known.

VDR polymorphisms:

The 1.25 dihydroxyvitamin D_3 steroid hormone plays an important protective role in the development of sporadic breast cancer. It can, however, elicit its effect only through its receptor, which encodes a transcriptive factor. The genetic modifications developing due to a point mutation in the VDR gene can cause severe gene activational defect. Thus it is evident that the VDR gene polymorhisms play an extremely important role in the efficacy of the inhibition of the proliferation of vitamine D tumuor cells.

If the point mutation takes place in a position, which is the site of division of a restrictive endonuclease then the length of the DNA fragment, digested with the given enzime, is different from the VAD-type. These two types can easily be detected by electrophoresis. These polymorhpisms are termed as restrictive fragment length

polymorphisms (RFLP). Several restrictive fragment length polymorphisms of the vitamine D receptor are known in the literature e.g. *Tru*9I, *Taq*I, *Bsm*I, *Eco*RV and *Apa*I.

*Bsm*I *polymorhisms:*

Hou *at al* showed that there is a significant difference between the allele distribution of patients and conrol groups by studying the relationship beetween *Bsm*I polymorphism and breast cancer: the risk factor of B allele carriers was higher. Similar results were presented by an American and also by a British case-control study, which demonstrated that BB homozygotes had increased risk factors. Others, e.g. Buyru *et al*, could not point out any relationship between the risk of developing *Bsm*I polymorphism and breast cancer in a Turkish population.

*Fok*I *polymorhisms:*

So far this is the only known protein polymorhism of the VDR gene and the results are rather different in connection with showing in what extent this polymorphism influences the frequency of certain tumour development. The protective effect of F allele was demonstrated e.g. in the cases of skin tumours, large intestinal tumours and breast cancer. Other studies showed that there is no significant correlation between sporadic breast cancer and *Fok*I polymorphism.

The role of the p53 tumour supressor gene in the development of breast cancer:

The p53 tumour supressor gene is one of the most intensively studied human cells. In the case of genom destruction p53 protein accumulates in the cell, promotes the transcription of the p21 protein and stops the cell cycle in the G1 phase until the damage is not repaired or induces apoptosis. Thus it prevents the division of cells damaged in their genetic matter. In the regulation of cell cycles the p53 protein elicits its effect as a trans-activator and activates the tarnscription of such genes, which inhibits growth and/or invasion.

Arg/Pro polymorphism:

The mutation of the p53 tumour supressor gene can be detected in 50% of the cases of human tumours. The majority of the p53 gene polymorphisms are in the area of introns, so it does not appear at protein level. Out of the exonal polymorphisms, the most remarkable one can be found in the area of exon 4 and area of amino acid 72, where the protein construction gets also

modified due to a guanin → cytosin substitution. One of the two alleles encodes arginine (CGC), the other proline (CCC).

I.2. The role of prevention

Although there has been a remarkable development in the field of tumour therapies in the last decades, except a few tumour types, tumour diseases cannot be regarded, with certaintity, as "curable diseases". In addition tumour therapies are rather expensive, the recently developed medicines are extremely costly.

Primary prevention should be approached from the side of risk factors. In the case of breast cancer each approach is regarded as a primary preventional approach, which targets to reduce oestrogen exposition or avoid other risk factors (e.g. carcinogenes from smoke).

Risk assessment (including the identification of high risk groups) is a means of prevention. Considering low penetration genetic factors is a means for developing it further and for making it more exact. The present thesis aims to investigate two of these low penetration factors, which are the allele polymorphisms of the p53 tumour supressor gene and vitamine D receptor.

Another useful approach for risk assessment can be to investigate gene expression changes. In the field of primary prevention, a further aspect of this doctoral thesis is to investigate the application possibilities of these expression changes.

Application of gene expression changes in prevention: c-myc; Ha-ras; p53

Several studies have shown that the expression of different genes in the tumour cells differ from that of those found in normal tissues. Since oncogenes and tumour supressor genes are key genes playing a role in the regulation of cell cycle, cell proliferation, differentiation and apoptosis it is not surprising that several authors have studied the expression of these genes. In the cases of different tumour types the c-myc, Ha-ras, N-ras, Erb-B2, p53 expressions, different from the ones found in normal tissues, have been presented. Further investigations, in accordance with the theory of 'field of cancerisation', have found onco/supressor gene overexpressions not only in the tumour cells but also in the healthy surrounding tissues, which seemed healthy both macroscopically and histologically. This finding indicates that gene expression changes may be applied to detect early involvment, also

in the stage when other factors, including mutations, do not warn us for a condition differring from the normal.

The protooncogene **c-myc** is one of the oncogenes discovered early. The gene encodes such a protein located in the nucleus, which functions as a factor of transcription. The myc protein is capable of making the cells in dormant stage proliferate again, i.e. it is able to continue the cell cycle. The protein forming a heterodimer with Max protein sequence specifically bonds to the DNA and, as a transcription regulator, inducing DNA synthesis it can lead to the development of transformed cells. The overexpression of c-myc has been presented in several tumours as well as in transformed cells and cell lines.

The **Ha-ras** (Harvey-ras) gene belongs to the ras gene family, the additional members of which are the Ki-ras and N-ras genes. The G-proteins encoded by the members of the ras gene family are the main elements of the intracellular signalling cascade, so they are involved in transmitting the proliferation signals reaching the cells. The ras proteins functions in the beginning part of the signalling cascade bound to the inner surface of the cell membrane. The end-effectors of cascades are the transcription factors located in the nucleus. The overexpression of ras genes may lead to the hyperactivity of the signalling system, i.e. it may result in transmitting proliferative signals in an exaggarated extent. Consequently, similarly to the previously discussed two genes, the overexpression of the Ha-ras gene has been detected in several tumours.

In the case of the **p53** tumour supressor gene the regulation primarily takes place through post-translational mechanisms. Consequently, mRNA level expression changes are also important and can be informative because some p53 transcription changes have been revealed in tumour tissues and cell lines.

The main point of **secondary prevention** is to diagnose the disease as early as possible, already in the asymptomatic period, when the chance for recovery is much better than in the case of already developed tumours, possibly infiltrating the surrounding tissues and giving metastases. A typical form of secondary prevention is to organise public screening tests.

Tercier prevention means the prevention of side-effects, maintainance or restoration of quality of life at as good level as possible as well as rehabiliation. In the cases of tumours, besides some other tasks, a crucial role of tercier prevention is to prevent the side-effects of cytostatic treatments and the development of metastases. In connection with cytostaic

treatments it is important to precisely know the biological phenomena of the tumour and to adjust the treatment to these and to the expected prognosis.

At present there are no reliable methods for the measurement and prognosis of side effects and contradictions (e.g. the development of possible secunder tumours). We cannot foresee those, in whom the treatment causes side effects with a greater probability, neither those, who can receive a greater dosage. The severity of acute symtoms does not necessarily help to answer the questions of late carcinogenity.

The present thesis proposed to study a new approach for the investigation of the carcinogenic effects of cytostatic treatment with the aim to demonstrate whether the risk of the second primary tumour induced by the therapy can be prognosed. This approach is based on the previously discussed gene expression changes. It was applied on white blood cells taken from periferal blood, as they were surrogate tissues. As continuation and development of the primary preventive investigation the gene expression of the c-myc, Ha-ras and p53 was examined in patients suffering from breast cancer not only at the time of drawing diagnosis but also after the treatment, grouping them whether the first intervention was chemotherapy (Cyclophosphamid, Methotrexat, Fluorouracyl = CMF) or operation. Thus, besides giving a prognosis for the expected efficacy of the therapy, we were able to indicate the possibly of developing side-effects as well as to measure the repairational capacity.

To make the results of the investigation more exact the effect of this chemotherapeutical protocol was examined in animal experiments to demonstrate whether it leads to onco/tumour supressor gene overexpressions in the animal experimental modell worked out earlier in the Department of Public Health at the Faculty of General Medicine at the University of Pécs, Hungary. If we can present that it does cause onco/tumour supressor gene overexpressions it will show that the results of the human experiment can be accepted and the results gained from the periferal blood are in accordance with the values measured in different organs in the animal experiments, i.e. they can be applied as their substitution markers.

II. Main Targets

1. We performed a case-control comparison in order to show whether the Arg/Pro polymorphism of codon 72 of the p53 tumour supressor gene, in the representive group studied, has an influence on the risk of breast cancer development. On the basis of the frequency of certain alleles we wanted to point out which alleles occur more frequently in patients suffering from breast cancer.
2. We wanted to demonstrate whether the *Bsm*I and *Fok*I polymorphisms of vitamin D receptor have an influence on the risk of developing breast cancer. Similarly to the above mentioned, we compared the frequency of *Bsm*I and *Fok*I alleles between patients suffering from breast cancer and controls.
3. On the basis of the relationship between the p53 tumour supressor gene and the gene polymorphisms of vitamine D receptor we wanted to present in what extent the risk of developing breast cancer increased in the group of suspected high risk allele carriers.
4. We measured gene expression changes from peripheral blood. In order to modell the risk of breast cancer and/or to show the exposition we compared the expressions of the c-myc, Ha-ras and p53 genes in patients suffering from breast cancer and in individuals without tumour.
5. We investigated the effect of Cyclophosphamid Methotrexat Fluorouracyl chemotherapy on gene expressions. Following the treatment of breast cancer patients (operation or cytostatic treatment using the CMF protocol) we compared the values of gene expressions with those before the treatment in order to demonstrate whether the development of the second primary tumour is in connection with the CMF treatment.
6. We investigated the effect of the CMF protocol on gene expressions in animal experiments. In the different organs of the experimental animals we measured the gene expression changes due to the CMF therapy in order to present in what extent the results of animal experiments supplement those gained from examining human peripheral blood.

III. Material and method

In the case-contol study 200 breast cancer patients were genotyped for the p53 and VDR genes and the allele frequency observed was compared with the allele distribution of the control population. The number, average age, sex and ethnic background of individuals in the control group were in accordance with the similar parameters of the group of patients.

The patients suffering from breast cancer were treated at the Department of Oncology of Baranya County Hospital, at the Department of Oncoradiology of Veszprém County Csolnoky Ferenc Hospital and at the Department of Oncoradiology of Vas County. The controls from the area of these counties were patients not suffering from cancer or healthy individuals coming for a regular screening. Since we did not want to investigate hereditary breast cancer but the factors playing a role in the deveopment of sporadic breast cancer we excluded those cases from the study, in which the family history and the results of the genetic examiantion indicated hereditary tumours or tumour syndromes. The mean age was 64.3 years (±7.2 years) in the group of patients suffering from breast cancer and 62.9 years (±8.2 years) in the control group. Both the breast cancer patients and the members of the control group were informed about the purpose of the study. Each participant took part in the study involuntarily. The members of the group of patients as well as the members of the control group were matched together on the basis of age and whether they were taking any hormone compound medicines.

Isolation of white blood cells

White blood cells were gained from 15 ml peripheral blood by repeated centrifugation using 0.84% ammonium cloride. Cenrtifugation had been repeated until the sediment lost its red or pink colour.

III.1. Polymorphism investigations

- **The investigation of the p53 *Arg/Pro* polymorphism**

The p53 *Arg/Pro* polymorphism was investigated by using an allele specific PCR. The method is based on the fact that the 3' end nucleotide of the 5' primer chosen for the PCR reaction correspond to the location of point mutation in the codon 72. Amplification is performed in the two tubes paralelly using the same 3' primer and one of the 5' primers, which

is different in its final base. On the basis of the presence or absence of PCR products the genotype can be determined.

3’ primer: GCAACTGACCGTGCAAGTCA

5’ primeres: ATGCCAGAGGCTGCTCCCCG (1)

ATGCCAGAGGCTGCTCCCCC (2)

Amplification is possible in the presence of *Arg* (CGC) in the case of number (1) primer and in the presence of *Pro* (CCC) in the case of number (2) allele. This way, by performing the electrophoresis of the PCR products developed in the two tubes in a parallele reaction, DNA, in a detectable quantity, will appear only in one tube in the case of a homozygote, while successful amplification can be carried out by both primer pairs in the case of a heterozygote.

DNA detection: Following amplification the whole quantity of the samples was run on a 2% agarose gel painted with ethidium-bromide.

- ## The investigation of the VDR polymorphism

The PCR-RFLP (restrictive fragment length polymorphism) method was applied to investigate the VDR gene polymorphisms.

- ***Bsm*I polymorphism:** *PCR*: to investigate the polymorphism at the 3' end of the VDR gene the 825-base-pair-length DNA fragment containing the division site of the *Bsm*I restrictive endonuclease was amplified by means of a PCR. The following primers were used for the polimerase chain reaction: 5’-CAACCAAGACTACAAGTACCGCGTCAGTGA-3’

  5’-AACCAGCGGGAAGAGGTCAAGGG-3’

RFLP: Then the 825-base-pair-length DNA fragment amplified by means of a PCR was digested using the *Bsm*I restrictive enzime and the whole quantity of the samples was run on a 1.5% agarose gel painted with ethidium-bromide.

When the division site of the *Bsm*I enzime misses the 825-bp-length product appears on the gel (B allele). When the division site is present (b allele) it is indicated by the fragmentation of the PCR product (650bp + 175 bp). On the basis of the fragment length appearing on the gel all the three genotypes can be identified.

- ***Fok*I polymorphism:** *PCR:* to investigate the polymorphism located in the area of exon II the 265-base-pair-length DNA fragment containing the division site of the

*Fok*I restrictive endonuclease was amplified by means of a PCR. The following primers were used for the polimerase chain reaction:

5'-AGCTGGCCCTGGCACTGACTCTGCTCT -3'

5'-ATGGAAACACCTTGCTTCTTCTCCCTC -3'

RFLP: Then the 265-base-pair-length DNA fragment amplified by means of a PCR was digested using the *Fok*I restrictive enzime and the whole quantity of the samples was run on a 2% agarose gel painted with ethidium-bromide.

The lack of the division site of *Fok*I defines the F allele, in which case the whole-length PCR product appears on the gel. When the division site is present (f allele) the enzime divides the amplified segment into a 196-base-pair and a 69-base-pair fragments. On the basis of this all the three genotypes (FF, Ff, ff) can easily be identified.

III.2. The investigation of gene expression changes

From the above hospitals 33 newly diagnosed breast cancer patients, patients undergone CMF treatment following 54 operations, patients not undergone CMF treatment following 31 operations and 50 healthy individuals as controls took part in the human experiment. Independently from the investigation of polymorhisms, the investigation of gene expression changes was performed in other patients. Total RNA was isolated from peripheral blood by means of the fonol-chloroform method. Then, by means of the Hoefer slot-blotter 10 μg RNA was placed on the Hybond N+ (Amersham) membrane following the protocol given in the Amersham ECL-kit. After this, following the protocol given by the producer, it was hybridised at 42 °C over night using the Amersahm ECL (enhanced chemiluminescence labeling) probe. The cloned gene probes inserted into plasmid of the Ha-*ras,* c-*myc, p53* and β-actin genes (American Type Culture Collection, Rockville, MD, USA) were cultured in an E. coli HB 101 bacterium strain at our department. As a control, the membranes were rehybridised by the constitutivly expressing β-actin gene. The chemiluminescence label was recorded on an X-ray film, which, following developing, was digitalised by a HP DeskScan IIC type scanner on a computer and the densities were evaluated by the Quantiscan 2.0 (Biosoft) programme.

III.3. Statistic methods

The estimated relative risk in relation with certain allelles and allele combinations was determined by statistical analysis. (odds ratio: OR) and 95% confidential intervall was calculated (CI). In the gene expression investigations the mean values of the groups were compared by *t-probe*. The calculations were performed by the help of the Epi Info for Windows (CDC, Atlanta) and the SPSS PC+ programmes.

IV. Results

The following results were recorded after the processing of the samples originating from the 200 breast cancer patients and the control population adjusted to the experimental group.

IV.1. Polymorphism investigations

The relationship between the p53 allele polymorphism and the risk of developing breast cancer

Table 1 presents the genotype distributions gained during the investigation of the *Arg/Pro* polymorphism in the patient and control group

	Patients	Controls
Arg/Arg	**105 (52,5%)**	137 (68,5%)
Arg/Pro	**60 (30%)**	56 (28%)
Pro/Pro	**35 (17,5%)**	7 (3,5%)
Total	200 (100%)	200 (100%)

Table 1: the p53 genotype distributions in the patient and control groups

The results show that, compared to the control group, the frequency of the rare *Pro* homozygotes remarkably grow, while the ratio of *Arg* homozygotes decrease among the patients. For the further evaluation of these data statistical calculations were performed, for which *Pro* homo- and heterozygotes were grouped together since both groups carry the allele indicating high risk (table 2).

	Patients	Controls
***Pro* carrier**	**95 (47,5%)**	63 (31,5%)
***Arg* homozygote**	**105 (52,5%)**	137 (68,5%)
Odds ratio (OR)	**1.97 (95% CI: 1.28-3.02)**	

Table 2: the ratio of *Pro* carrier individuals in the patient and control populations

Our investigation showed that the risk of *Pro* carriers increase since the presence of the *Pro* allele was more frequent in the group of breast cancer patients. Analysing the relationship between the polymorphism p53 *Arg/Pro* and the risk of developing breast cancer a statistically significant result was gained: the rare *Pro* allele carriers have a 1.97 odds ratio to develop the disease (OR:1.97; 95% CI:1.28-3.02).

The relationship between the VDR allele polymorphism and the risk of developing breast cancer

Table 3 presents the genotype disrtibutions gained during the VDR allele polymorphism investigations.

	Patients	Controls
***Fok*I polymorphism**		
FF	**63 (31,5%)**	68 (34%)
Ff	**114 (57%)**	101 (50,5%)
Ff	**23 (11,5%)**	31 (15,5%)
***Bsm*I polymorphism**		
BB	**51 (25,5%)**	29 (14,5%)
Bb	**92 (46%)**	115 (57,5%)
Bb	57 (28,5%)	56 (28%)

Table 3: Genotype distribution of the groups of patients and controls considering the investigated VDR polymorphisms.

In the case of *Fok*I polymorphism the allele distributions gained by genotyping the control group do not remarkably differ from the results gained from the investigation carried out among European women. The results of the *Bsm*I polymorphism investigation showed that the frequency of B allele in the control group was 43%. Investigating the relationship between the VDR polymorphisms and breast cancer the following results were gained by analysing the data (table 4).

The investigated allele polymorphisms	Odds Ratio (OR)	95% confidential intervall
***Fok*I (F homozygote)**	**0.89**	0.58-1.39
***Bsm*I (B homozygote)**	**2.02**	**1.18-3.46**

Table 4: The effect of the investigated VDR polymorphisms on the development of breast cancer

On the basis of the literature we had expected the protective effect of the FF genotype against the development of breast cancer but we were not able to show any correlation. Although there are less homozygotes among the patients the result was not significant statistically.

In the case of the *Bsm* I polymorhism, however, statistically significant risk increase was demonstrated in connection with the BB genotype. The risk factor of the homozygote women carrying high-risk alleles are 2.02-times higher than that of b homo- and heterozygotes.

The investigation of the correlation among genes

In the cases of individual polymorphisms the simultaneous presence of high risk genotypes was investigated in the patient and control groups to study the gene-gene correlation. Those individuals were involved in the investigation from each group, who were *Pro* allele carriers, which proved to be a risk increasing factor on the basis of the results of the p53 polymorphism investigation, and also, considering their *Bsm* I genotype, BB homozygotes, which is also a high risk factor.

Table 5 presents that those individuals, who carry high risk allele combinations of genes partly overlapping in their functions are remarkably over represented in the patients group compared to the control population. The relative risk factor shows a 4.87 increase in women with such genotype combination.

Examined genotye	Occurrence		OR	95% CI
	Patient group	Control group		
P53 Pro carrier	95 (47.5%)	63 (31.5%)	1.97	1.28-3.02
BsmI BB	51 (25.5%)	29 (14.5%)	2.02	1.18-3.46
***P53 Pro* carrier+ *BsmI* BB**	**30 (15%)**	**7 (3.5%)**	**4.87**	**2.02-13.42**

Table 5: simultaneous occurrence of high risk genotypes, correlation among genes

Another approach to investigate the correlation among genes is to examine in what ratio the genotype indicating high risk considering one of the genes appear simmultaneously with the high risk allele of the other gene in the patient and control group. For this investigation the *Bsm* I homozygotes were selected from each group and we examined which p53 alleles the high risk VDR genotype combines with in the patient and control group.

There were 29 BB homozygotes in the control group, while 51 in the group of patients suffering from tumour. The following table presents their p53 genotype distribution.

p53 genotype	BB homozygotes	
	Patient group (51)	**Control group (29)**
Arg/Arg	**21 (41.2%)**	22 (75.9%)
Arg/Pro	**23 (45.1%)**	7 (24.1%)
Pro/Pro	7 (13.7%)	—

Table 6: p53 genotypes of BB homozygotes in the patient and control groups

These data show that the combination of the protective *Arg*/*Arg* genotype and the BB *Pro/Pro* genotype occurs only in the patient group.

While the BB homozygotes in the control group, considering their p53 genotype, are mainly (76%) *Arg* homozygotes and in a smaller extent (24%) *Arg/Pro* heterozygotes, the ratio of *Arg* homozygotes decreases (41%) and the BB genotype is mainly accompanied by one (45%) or two (14%) *Pro* alleles in the control group.

Our investigation showed that there is a significant correlation between certain, entirely general in their occurence, variations of polymorpf genes and the risk of developing breast cancer. Although certain high risk alleles increase the individual risk only in a small extent the individual difference in sensitivity resulting from gene polymorphisms, as the

investigation of interactions among genes demonstrated, together or in interaction with each other (and possibly also with some environmental facctors) influence the probability of developing cancer in a greater extent.

IV.2. The investigation of gene expression changes

The results of the human experiment

Figure 1 pressents gene expressions gained from peripheral blood.

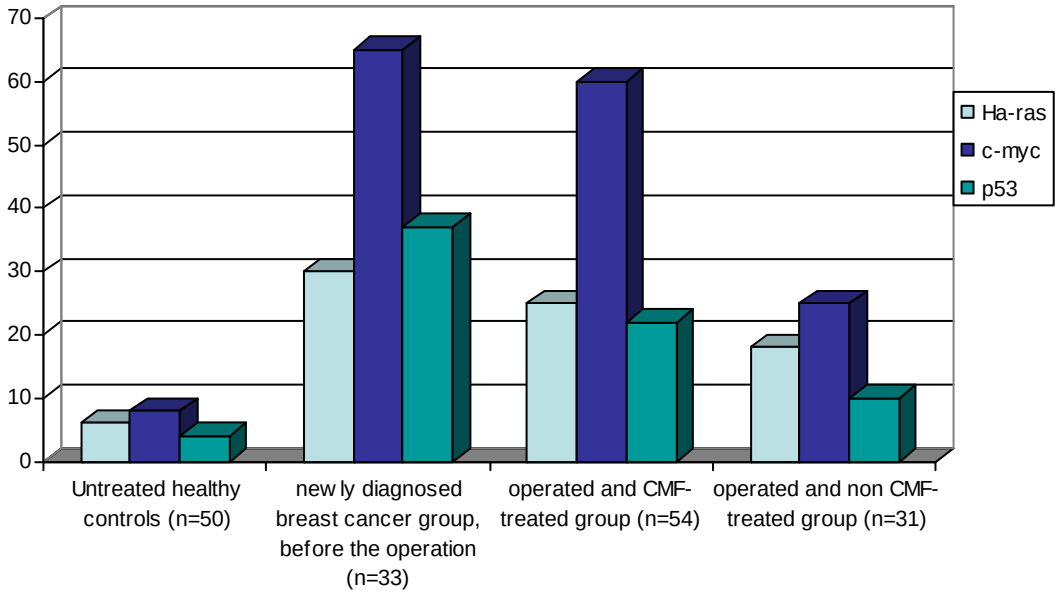

Figure 1. The expressions of the examined genes in patients suffering from breast cancer and in the control group.

The expressions of the examined genes were relatively low in the group of healthy controls. In the group of newly diagnosed breast cancer patients the expressions of all the three genes were statistically significantly different from those of the controls. These data clearly show the value and applicability of gene expression changes. The results alone,

however, do not explain what is in the background of gene overexpressions measured in the cells isolated from peripheral blood.

In patients undergone operation, in the cases when they had not received any cytostatic treatment until the time of investigating the gene expressions, the gene overexpressions remarkably decreased compared to the group of newly diagnosed patients, while they presented higher values in the cases of all the three genes in the group of untreated healthy controls.

Finally, a minimal decrease could be detected in the group of patients undergone CMF treatment following operation compared to the newly diagnosed group.

Comparing the last two groups we can see that the only difference was the CMF treatment. This showed that the expression of all the three examined genes was remarkably higher (in the cases of c-myc and p53 genes more than the double) in the group of patients undergone CMF treatment following operation compared to the group of patients not receiving the CMF protocol.

The results of the animal experiment

Figure 2 presents the results.

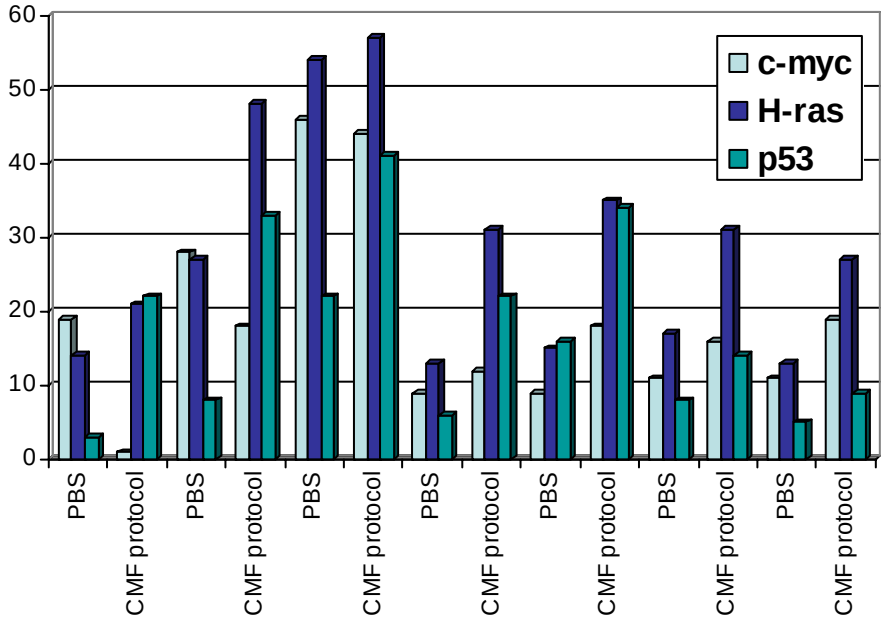

Figure 2. Gene expression changes due to the CMF protocol (in CBA/Ca mice)

The expression of the Ha-ras oncogene was increased without any exemptions in every organ compared to the controls. This increase was statistically significant in the spleen, the kidneys, the thymus, the thyroid glands and the bone marrow, while in the liver it was a bit lower and in the lungs it was relatively decreased.

The p53 tumour supressor gene was also over expressed in every organ. Here the expression increase found in the bone marrow was the only one, which did not reach the statistically significant level.

The expression of the c-myc oncogene showed a more various picture than the previous the genes. Lower gene expressions were detected in the liver, the spleen and the lungs in the group of patients treated with CMF than in the control group treated with physiological sodium-chloride solution. In the case of the lungs the difference was minimal, while in the cases of the liver and the spleen it was statistically significant. In the other four examined organs the CMF protocol enhanced the expression of the c-myc gene, which was statistically significant in the cases of the thymus and the bone marrow.

The results showed that the overexpressions of the Ha-ras and p53 genes are capable of indicating the potential carcinogenic effect of the CMF protocol, while the c-myc gene, in this respect, is a less usseful biomarker. The results also suggest that the evaluation should organ specifically be performed since the basic gene expressions as well as the gene expression patterns can be different in every organ.

V. Discussion

In the investigation two typically individual sensitivity factors - the allomorphisms of the p53 tumour supressor gene and the vitamine D receptor - were studied with respect to their effect elicited on the development of breast cancer. The results demonstrated that two factors out of the examined three (the VDR *Bsm*I and p53 *Arg/Pro* polymorhisms) can be responsible for the developmnent of the disease.

In the case of the vitamine D receptor, not even the literature explains why and by what mechanism this polymorphism influences the risk of developing the disease. Since the polymorphism is located in the area of introns (between exons 8 and 9) it does not change the amino acid sequence of the encoded protein. As the *Bsm* I polymorphism does not control the

construction of the receptor protein, the protein encoded by the two alleles are functionally identical and the protective effect of b allele is achieved by increasing the stability of the transcripted mRNA. The hypothesis referring to the effect mechanism, however, has not been underpinned by exact molecular biological and molecular epidemiological data yet.

On the other hand, there are several data known in connection with the 72 codon Arg/Pro polymorphism of p53 as well as with the different functioning of the encoded proteins. The risk increasing effect of p53, which is quite rare in the Caucasian population is probably related to the less effective apoptosis inducing ability of the protein containing proline in a polymorph site. Consequently, cells, which are injured in their genetic matter in such extent that the repairation of irregularities becomes impossible and which should be eliminated to prevent tumour development can survive in a greater probability in *Pro* carrier individuals. In accordance with this, and also with the results of our investigation the *Pro* allele carriers belong to the high risk individuals.

Correlation between the low penetration genetic factors can be detected in our sample. While the risk factor of high risk allele carriers were about the double of that of low risk allele carriers examining both polymorphisms separately, in the case of the simultaneous occurrence of the two factors the risk was 5 times higher. Such interactions can explain the epidemiological phenomenon of the development of sporadic tumours, namely, that having the same exposition not the same effect and disease develop in different individuals. Investigating additional low penetration genetic factors is needed to make individual risk estimation more exact. Today its technical background is supported by the spread of the microarray techniqe.

Consequently, further investigations are needed to be able to estimate the inclination to developing tumour diseases more precisely. Besides the investigation of genes involved in developing individual inclination, it is important to understand the correlation among genes and the interactions of the gene environment. Research aiming to explore these correlations seems to bring practical benefit. In the view of the individual risk factors the development of certain diseases may be prevented by appropriate way of life, possibly by chemoprevention, and frequent medical screening. To establsih individual risk estimation and personal prevention on the basis of genotype examination can be an opportunity in the distant future. This, besides the present molecular genetic screening examinations, will hopefully introduce new approaches in the prevention of breast cancer despite possibly rising some new ethical issues (Oláh, 2003).

The expression changes of onco/tumour supressor genes can be applied as entirely different biomarkers. These have already been used in pathological diagnostics as prognostic markers. The present thesis, however, cancentrated on their applicability in the prevention.

Unfortunately the curability of tumours is about 50% in Hungary in ideal circumstances. That is why we should focus on primary prevention, the efficacy of which can even be 25-30%. Besides reducing morbidity, the consequent mortality should be decreased. Considering effective primary prevention, however, public and more and more preferably individual risk assessment and identification by molecular and predictive epidemiological biomarkers are extremely important far before the development of the tumour. Intervention and monitoring its efficacy by appropriate biomarkers are similarly crucial. Besides screening methods, the application of biomarker panels should be spread more widely, which can assist prevention at any level.

The majority of diagnosed and treated patients are lost due to metastases and contradictions developing because of late detection and weaknesses of therapy since, while diagnostics and pathology have been following a molecular approach therapy could not do the same. Prevention of metastases and cytostatic treatment induced second primary tumours are crucial in the treatment of breast cancer and the follow up of patients. It is a key question because the curability of secundary tumours are more complicated, the course of disease is shorter and there are more complications, which places a great burden both on the patients and the health care system.It is well known that the chemotherapeutical agents cause second primary tumours in 1-5%. So far the second tumour inducing effect of alkylates has been investigated the most thorougly, especially in haematological pictures (Non-Hodgkin lymphoma, leukaemia).

Such new methods should be introduced, besides the ones, which have already been proved to be adequate, which, on the basis of modern molecular biology and molecular epidemiology, help to detect risk by non-invasive interventions and examinations. Moreover, results assisting diagnostics and providing prognosis for the patients can be gained.

It seems that the properly selected gene expression changes as biomarkers at a molecular level, following further detailed investigations, will be appropriate for these tasks. Up to present methodologies based on gene expressions have assisted the struggle against malignant tumours as diagnostic methods(Kopper, 2002). Their advantage is that these biomarkers are capable of detecting epigenetic effects. Considering the field of application described here, their main disadvantage is that they are not specific enough and they cannot

be evaluated in an exact way at an individual level. Hopefully, in the long run the establishment of appropriate disease or risk factor specific gene panels will assist the evaluation of individual risk. Although the gene expression changes are not diagnostic markers or biomarkers, which are appicable for screening examinations, it seems that they are able to detect the potentially carcinogenic expositions or their early effect. Former animal experiments showed that overexpressions correlated well with the late development of tumours, which is an additional reason for the application and further study of these biomarkers.

The cause or the origin of the gene expression changes measured from peripheral blood can be questionable. It can be assumed that tumour cells are discharged into the blood stream and these cells, mixing with white blood cells on isolation, lead to a generally high expression. The presence of the necessary quantity of tumour cells for this process, however, seems unrealistic. It is possible that the changes induced by the presence of tumours (signal transmitting molecules, inflammation mediators, metabolic products) lead to the altered matter of cells in the peripheral blood, which can be followed by the examined gene expressions. It should not be neglected that the main ratio of lymphocites are in a repopulation phase and the long-life lymphocites can reveal the expositions suffered, giving information about the expositions suffered in other kinds of cells (hormonal and non-hormonal effects, exogenic expositions) as well as about the repairation ability of the organism. The increased gene overexpressions due to cytostatic treatment can similarly be explained..

First a theoretical explanation should be found in the field of gene expressions measured from peripheral blood, then it should be proved. This, however, does not indicate that the investigation of these biomarkers are not necessary. On the contrary, in the view of the so far encouraging results, further investigation is needed, especially in the field of histology and by considering the different stages, in order to find their place, as widely as possible, among the biomarkers in the tumour prevention at molecular level.

The above investigations are not primary privention applications. They can serve, however, as a base to introduce the application of gene expression changes in the course of primary prevention since they can be the early biomarkers of tumour development or abnormal functioning.

VI. Summary of novel results

1. In the subpopulation investigated the 72 codon polymorphism of the p53 tumour supressor gene significantly influenced the risk of developing breast cancer. It was higher among the Pro alle carriers than among the Arg allele carriers.

2. The BsmI polymorphism of vitamine D receptor also influenced the risk of developing breast cancer. The B allele proved to be a high risk allele.

3. The simultaneous occurence of the two high risk alleles (the p53 Pro and BsmI B alleles) increased the risk by more than the double.

4. The FokI polymorphism of vitamine D receptor had not a significant effect on the risk of developing breast cancer in the investigated group.

5. In the cells isolated from the peripheral blood of patients suffering from breast cancer the expressions of the c-myc, Ha-ras and p53 genes were significantly higher than those of the healthy controls.

6. In patients suffering from breast cancer the above gene overexpressions remarkably decreased following operation.

7. The CMF protocol applied postoperatively inhibited the above gene expression decrease.

8. In the animal experiments the CMF treatment, in the examined organs (liver, spleen, lungs, kidneys, thymus, thyroid glands, bone marrow), enhanced the expressions of the three investigated genes (except in the lungs, liver and spleen in the case of the c-myc gene), i.e. the gene expression changes proved to be appropriate biomarkers of expositions with potentially carcinogenic effects.

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