

GENES - FROM LABORATORY TO CLINICAL PRACTICE IN ONCOLOGY

INTRODUCTION

Genes, individual units of inheritance, carry detailed instruction for all cells in the organism by encoding specific biologically active products. The sequence of DNA strands determines how the instructions are read and interpreted into the production of polypeptides. Mutations (changes in the structure of DNA) can lead to inactivation of protein or to the aberrant function of produced protein. Although in the past it was believed that the etiology of cancer is solely connected with toxic or viral agents, recently it has become apparent that most cancers develop as a result of a combination of genetic and environmental factors. It is accepted that cancer is a disease of genes on the molecular level (1). Also, estimation is that alterations of 3 to 7 genes are necessary for malignant transformation of the cell. The majority of these genes belong to oncogenes, tumor suppressor genes and repair genes. In some cases, genetic predisposition to a particular type of cancer is the strongest risk factor for an individual. One of the exciting expectations of the human genome project (HGP) was to find and define the genes connected with cancer development and therapy. In that way the term, "geneticization", coined in 1991 by Abby Lippman (2) and used to characterize the impact of advances in genetics on the population at large, would have full recognition. Adversely to great expectations from genetics for diagnosis and treatment of cancer, deficiency of training and knowledge of physicians and clinical oncologist is evident. One of the reasons is the fast development of new diagnostic tests and therapy based on genetic knowledge and the other, maybe more important, is that these advances are not followed by adequate clinical application. For that reason, the impact of molecular genetics to every-day clinical practice in oncology will be discussed in this article.

MOLECULAR DIAGNOSTICS OF CANCER

Molecular diagnostic procedures

The target molecule for molecular genetic analysis such as DNA is very long, composed of millions of base pairs and practically it was impossible to detect alterations in one or few nucleotides with classical genetic methods. In the early 70-ties, the group of bacterial enzymes known as restriction endonucleases was found to cleave DNA in a sequence specific, and therefore, predictable manner into defined pieces. Most restriction enzyme sites are defined by 4–6 base pairs, but some can be much longer. Usually, the longer the site, the more rare is its occurrence in the genome. The enzyme cuts on the both strands of DNA within a restriction site resulting in DNA ends that are blunt, or to form cohesive i.e. complementary ends. These enzymes enabled for the first time the targeted „manipulation“ with DNA molecules resulting in the first restriction maps of different plasmids. Although it was a great breakthrough in the molecular biology for

diagnostic purposes, one must have in mind that most DNA and RNA molecules are very long, they are present at low levels and are difficult to detect and analyze in the complex population of molecules by standard biochemical procedures (3). For that purpose, the future of single stranded DNA or RNA to associate with complementary RNA or DNA owing to formation of hydrogen bonds between A-T and G-C pairs was exploited. The procedure by which a labeled DNA or RNA (probe), is used to detect its specific complementary molecule is known as hybridization. The molecular biology technique in which DNAs are separated by size via gel electrophoresis and transferred to a membrane for hybridization is called Southern blotting, and for RNA is termed Northern blotting. Double stranded DNA must be denatured prior hybridization by heat or chemicals. The conditions for binding of the probe are determined by the number of G-C versus A-T pairs, and the number of continuous perfect matches between sequences. Although these methods are highly sensitive and specific they are not quite good for everyday practice because they require relatively highly educated personnel and large amount of high molecular weight DNA which has been extracted from fresh/fresh-frozen tissue or cell suspensions. These limitations are overcome by application of polymerase chain reaction (PCR) techniques (4), which can be defined as in vitro amplification of targeted DNA sequence. With this technique, a sequence of DNA can be amplified up to 106 to 109 times so it may be analyzed directly for point mutations or polymorphisms, and then, if necessary, sequenced. The requirement is that nucleotide sequence of targeted sequence is known. Thermostable DNA polymerase (Taq DNA polymerase) catalyzes copying of DNA in proliferating bacterial cells, and also in vitro. For starting the process template DNA, primers (short sequences of DNA complementary to the flanking regions of targeted sequence), four deoxynucleotides (dATP, dCTP, dGTP, dTTP) and Taq DNA polymerase are required. With appropriate conditions, in a few hours, it is possible to study DNA from before birth to even after death. The DNA template can be retrieved from many sources including smears, hair roots, blood spots, paraffin-embedded tissues and even single cells. The amplified DNA can be visualized on a size fractionated gel, or analyzed further by restriction endonuclease digestion, hybridization with labeled highly specific probes or sequenced. Sequencing of DNA samples (determination of the base sequence of the isolated DNA) after sequencing of complete human genome and after introduction of automated capillary electrophoresis in laboratory practice becomes now available to clinical laboratories (5). The latest automated sequencing systems have the capability to run, detect and analyze 96 different samples simultaneously. DNA sequences from different sources have been obtained and are available through electronic access from

EMBL database, DDBJ and Gene Bank sequence library. Because of the wide variety of applications in which PCR is now being used, it is probably impossible to describe a single set of conditions that will guarantee success in all situations. For that reason, in the everyday use there are different PCR variants such as reverse transcriptase PCR (RT-PCR), asymmetric PCR, multiplex PCR, touch down PCR, inverse PCR, differential display PCR, allele specific PCR, in situ PCR, etc. RT-PCR which is used for detection of RNA targets is used more than other PCR variants in clinical practice. In this reaction cDNA (DNA complementary to RNA) is firstly created using a reverse transcriptase enzyme and then subsequently amplified by thermostable DNA polymerase. Originally the method used a two-step procedure, firstly reverse transcription and secondly DNA amplification. The development of polymerase which combines reverse transcriptase and DNA polymerase activity (rTth polymerase), excludes the need for a two-step reaction. By analyzing the presence/absence of rRNA one can get conclusions about gene activity in the cell population of interest. Comparison of the end product of RT-PCR reaction of the examined gene with the gene that is constitutively expressed (like β -actin gene) can give information about transcriptional activity of that gene, but it is only semi-quantitative measuring (6). Currently, the best way to measure gene expression is usage of real time PCR technology that combines thermal cycling, fluorescence detection and application-specific software to detect accumulation of PCR products after each cycle in the single tube reaction (7). At a certain point during cycling, the product accumulates enough to increase fluorescence above background noise and correlates to the amount of starting copies within a PCR. Quantitative results are available immediately upon completion of PCR offering enormous time savings and greater sensitivity of reaction (8). A common method of quantification by real-time PCR is using SYBER Green as a fluorescent indicator of double stranded DNA production. SYBER Green detects PCR products by directly intercalating into any double stranded DNA. An alternative to the using of SYBER Green is real-time PCR using 5' nuclease assay probes, based on the hybridization of a labeled probe to the PCR product. Real-time PCR also can be used for detection of mutation (9) and single nucleotide polymorphism (SNP).

A vast majority of human cancers display multiple gene deletions and amplifications, genetic instability, gene rearrangements and hypermethylation making the final effects of these alterations difficult to study. Traditional methods were limited to examining a few genes at a time. In the past several years, a new technology promises to monitor tens of thousands genes simultaneously on a single chip (10). Terminologies used to describe this technology include: biochip, DNA chip, DNA microarray, gene array and gene chip. The underlying principle of gene chip is hybridization of nucleic acid polymers immobilized on a solid surface (11), as probes for complementary gene sequences. The most often used

variant of gene chip technology consists of an array of oligonucleotide (20–25-mer oligos at least in triplicate) synthesized either in situ (on-chip) or by conventional synthesis followed by on-chip immobilization. The array is exposed to labeled sample DNA (cDNA), hybridized, and identity/abundance of complementary sequences are determined. This chip can be applied for identification of sequence (gene/gene mutation) or for determination of expression level (abundance) of genes. Commonly cDNA microarrays are queried simultaneously with cDNAs derived from experimental and reference RNA samples that have been differentially labeled with two fluorophores to allow quantification of differential gene expression. Such gene chip must have hybridization standards, genes expression controls (constitutive genes) and gene probes of interest. Expression profiling techniques have been used to monitor simultaneously the expression of thousand of genes from human tumor samples (12). Although, they are relatively easy to use, the analysis of obtained data can be long and hard.

Molecular diagnostics of cancer in practice

Molecular diagnostic procedures can be used for screening and early diagnosis of cancer (13). A variety of mutations, microsatellite instability, epigenetic and chromosomal changes are targets for molecular diagnostics of cancer (14, 15). Mutations that can alter the function of genes include insertions, deletions, duplications and inversions of genes, and substitutions of single base pairs. Mutations outside the coding sequence can affect transcription, translation and mRNA splicing and processing. Genetic instability is an intrinsic property of cancer cells that frequently results in sequence alterations in microsatellites (tandem repeats of DNA nucleotides that occur abundantly and randomly throughout the human genome) or as a result of gross chromosomal changes (aneuploidy, translocation, rearrangement, deletion). For identification of chromosomal changes fluorescent in-situ hybridization (FISH) technique can be used (16). Epigenetic changes are defined as heritable alterations in gene function that are mediated by factors other than changes in the primary DNA sequence. DNA methylation alters gene expression in the way that promoter sequences of the actively transcribed genes have low levels of methylated DNA, whereas transcriptionally silent genes are often highly methylated at their 5' end. Therefore, it is possible that by DNA methylation function of tumor suppressor gene is decreased, while expression of proto-oncogenes could be increased (17). Another epigenetic mechanism is genomic imprinting, a reversible modification of DNA that causes differential expression of maternally and paternally inherited homologous genes. Loss of imprinting involves the expression of genes that are usually not expressed in a manner that is specific for the parent of origin. In that way normally silent copy of growth-promoting gene can be activated and silenced by normally transcribed tumor suppressor gene (18).

Clinical screening of asymptomatic individuals by using molecular markers can detect disease in its pre-clinical phase. For that purpose microsatellite instability (colon cancer, lung cancer), hypermethylation of gene promoter sequences (lung cancer, oral cancer, gynecological cancers), detection of carcinogenic viruses (high risk types of human papilloma viruses in the cervical intraepithelial lesions), oncogene and (K-ras in the colon and pancreatic cancer, lung cancer) tumor suppressor gene mutations (p53 in bladder cancer, lung cancer) can be used (19, 20, 21). Molecular genetic probes in clinical screening of malignant diseases are mostly demonstrated by different case reports like it was shown in one that p53 mutation could be detected in the urine sample nine years before the diagnosis. Similarly, genetic alterations were found in colonic washing, pancreatic juice, sputum, cervical swabs and bronchial lavage. However, the usefulness of particular molecular methods is limited by finding the same alterations in healthy persons which never develop cancer, and also one must have in mind sensitivity, specificity, positive predictive value of the tests and acceptability of the test to patients (22–25).

Great sensitivity of molecular diagnostic procedures and early appearance of genetic alterations in the process of cancerogenesis were promising for discovery of new, reliable diagnostic tests in oncology. Until now, we have had only few examples that molecular diagnostic tests satisfied diagnostic criteria. The main reason is that genetic alterations are not specific to particular tumor tissue. On the contrary, mutations in the same genes are found in different tumors. Also, genetic alterations are rarely 100% present in particular tumors making impossible to use data of presence/absence of genetic alteration as reliable diagnostic criteria.

Currently, situation is much more promising in hematology (26) where rearrangements of the genes for antigen receptors in lymphocyte malignancies and bcr-abl translocation in chronic myeloid leukemia (CML) are used as diagnostic tests.

Conventional laboratory diagnosis of B and T cell proliferations is based on histology or cytology, while clonality of B or T cell populations is established mainly by immunophenotyping. However, lymphomas, especially at early stages of development, can be very difficult to resolve from reactive lesions, even with the aid of immunophenotyping. The discovery of antigen gene rearrangements and the application of molecular probes (27) for these genes have been shown to be of value as they can demonstrate monoclonality, assign a disorder to either the B- or T-lymphocyte lineage, and enable the detection of a small number of neoplastic cells. Rapid clonality analysis of almost any clinical sample can be carried out by PCR amplification of randomly assembled different variable (V), sometimes diversity (D), and joining (J) gene segments of Ig heavy chain gene (IgH), immunoglobulin light chain gene for B lymphocytes and α , β or γ , δ gene rearrangement of T cell receptor (TCR). The complementarity determining region III (CDR3) of VH gene segment formed by VDJ junction can be

amplified by PCR with primers that bind to consensus gene regions in IgH V framework regions. Primers have been designed to bind and amplify the most constant sequences or consensus regions within framework (FR) I, FR II or FR III to the J regions. Only complete VDJ rearrangements with the V and J sequences in the right orientation are amplified (28). Monoclonal populations yield one or two dominant products, whereas polyclonal samples yield a wide range of product sizes that appears on gel. In that way, sensitive PCR-based techniques for detection of clonally rearranged immune genes might become useful and broadly applicable diagnostic tools for monitoring of patients with B and T-cell malignancies (29). Results to date suggest that clonality analysis of IgH gene rearrangement is reliable using paraffin embedded samples in approximately 70–80% cases (30). Other reports suggest that up to 96% of low grade B-cell, non-follicular lymphomas and 82% of high or intermediate grade lymphomas can be amplified by using multiple primers (31,32). In addition, this technique has a great potential in tracking minimal residual disease in lymphomas and leukemias (33, 34) and for monitoring clonal evolution in acute and chronic lymphoblastic leukemias and lymphomas (35) and also by detecting clonal rearrangements of T-cell receptor for differential diagnosis and monitoring of T-cell lymphoma (36). The use of CDR3 as a clonal marker of multiple myeloma showed excellent sensitivity as it was demonstrated that circulating malignant cell could be identified in the majority of patients at a frequency of 0.001% to 1% (37). Because of its speed and simplicity, the PCR analysis is given priority when diagnostic assistance in a difficult case is required (29, 34). But, it must be kept in mind that molecular test results have to be viewed in the context of morphology and immunohistochemistry. The detection of a clonal lymphocyte population in clinical samples is always abnormal but still it should not be considered proof of malignancy.

Molecular staging of cancer

Accurate determination of the extent of local, regional and distant spread of a cancer (clinical staging) is assessed by the histopathological study of cells found at the margins of tissue removed during surgical resection of cancer, and cells found in the draining lymph nodes of the cancerous area. Although sensitivity and specificity of histopathological methods are improved, it is hard to expect that by using these methods it will be always possible to detect a small number of cancer cells. Molecular characterization of cancer cells in the resected tumorous tissue gives chance to find the same genetic alterations even in the single malignant cell (38–43). Molecular based staging has been evaluated in the study of 25 patients with squamous carcinoma of the head and neck who, on the basis of a negative histopathological assessment, have had complete tumor resection (44). When mutations in p53 gene were examined 13/25 resected margins were „positive“, and also in one quarter of patients lymph node cancer cells were detected, al-

though, they were initially considered to be „negative“ by histopathological assessment. None of 12 patients whose tumor margins were assessed to be negative by molecular staging have not developed local recurrence of the cancer, while 5/13 patients whose margins were assessed to be positive developed local recurrence. Similarly, in colorectal, lung cancer, head and neck cancers and pancreatic cancer mutations of K-ras and p53 genes have been used to identify the presence of cancer cells in lymph nodes and for detection of cancer cells in the systemic circulation. There are many examples in which genetic alterations were examined in resected tumorous tissue, tumor margins, lymph nodes and in the blood (usually by RT-PCR, cells of ectodermal origin were detected) aimed to introduce new staging or sub staging of tumors, but real breakthrough in this area is expected from introduction of genechip in oncology. Currently, there are many data indicating that synchronous analysis of structure or expression of a few thousand of genes gives possibility for new staging of tumors, for example, to differentiate at the time of diagnosis indolent from aggressive lymphomas. More optimistic expectations are that in the near future we'll have completely new, molecular staging of tumors (44–50).

Prognostic and predictive value of molecular testing

Taking into consideration that p53 is most commonly mutated gene in human malignancy, it is clear then why prognostic value of p53 has been investigated in many studies worldwide. Although the results of these studies have sometimes been conflicting it is possible to conclude that p53 mutation in breast, colorectal, lung and head and neck cancer can represent an independent marker of early relapse. In cells where DNA damage has occurred, e.g. after gamma irradiation or after chemotherapy, p53 accumulates and cell proliferation is arrested. This mechanism is important for preventing reproduction of DNA damage and giving the cell time for DNA repair, or for eliminating those cells in which DNA repair has not been successful (51). Cells that have p53 mutated or bind onto other cellular and viral proteins, are not arrested in G1 phase and do not undergo apoptosis. Such cells are consequently genetically less stable and may develop malignant cell clones. Several chemotherapeutic agents (cisplatin, etoposide, teniposides, DNA alkylating agents, macromolecular synthesis inhibitors, dexamethasone, topoisomerase I and II inhibitors) and radiation have been shown to be able to induce apoptosis in a number cell lines in vitro (52). Malignant cells with wt p53 arrest cell cycle and enter apoptosis, while cells with a mutated p53 cannot enter apoptosis. In that way, the damaged DNA is replicated. As a consequence, progressive multiplication of mutated cells and appearance of clones of more and more aggressive malignant cells take place. Surprisingly, tumor cells with impaired p53, at the same time can become less viable due to the fact that these cells more likely enter S phase and mitosis bearing high levels of

unrepaired DNA. Moreover, the cycling fraction of p53 deficient cell population is often high, and, if drug is one that preferentially acts during S phase or mitosis, lethality would be increased. The consequences of altered p53 status for drug efficacy in cancer therapy are, therefore, difficult to predict. Recent study have indicated that group of the patients with II and III stage of head and neck carcinoma, had shortened disease free interval and overall survival if mutation in the p53 gene was found compared to the group of patients without mutation (53–57). Mutations in p53 gene are predictive of poor survival in patients with colorectal carcinoma, while K-ras mutations have been shown either to be more frequent in patients with recurrent disease or to be an independent marker for poor prognosis (58).

Studies conducted up to now on various model systems (lymphomas, ALL, breast cancer, lung cancer) have shown that the ability of cytostatics to induce apoptosis of malignant cells represents a positive prognostic parameter in the treatment of tumors. Some cells die more readily than others, as a result of the balance of pro- and anti- apoptotic molecules (e.g. from bcl-2 family) which together sets a particular cell „threshold“ for death. The deregulation of bcl-2 (bcl-2 oncogene was first identified as a gene over-expressed in human follicular B-cell lymphomas, following reciprocal chromosomal translocation) and its partners like bax and bcl-x can protect cancer cells from apoptosis upon anticancer drug treatment. Although clinical studies paradoxically suggest an association between bcl-2 expression and a good prognosis for cancer patients, the clinical relevance of bcl-2 family is still under analysis (59–62).

Real breakthrough in assessing the prognostic/predictive significance of the molecular marker were results of the studies of breast cancer that confirmed that amplification of the her-2/neu receptor oncogene was associated with earlier than average relapse and reduced overall survival. In breast cancer her-2/neu gene is amplified in 15–20% of cases, conferring poor prognosis. Nowadays, amplification of this oncogene is accepted as an independent prognostic marker for breast carcinoma patients, and also results of different studies showed that this molecular marker could predict response to treatment (63, 64). Another molecular marker that has already been introduced in everyday clinical practice is bcr-abl gene that represents reciprocal translocation between chromosomes 9 and 22 (Philadelphia chromosome). Quantification of bcr-abl fusion gene transcript is essential for predicting results of CML therapy and tracking minimal residual disease at the molecular level. Chronic lymphocytic leukemia (CLL) is a heterogeneous disease. In the majority of cases patients remain asymptomatic for many years without any treatment, whereas some will develop symptomatic CLL with poor outcome. For better treatment of this group of patients, it is of the outmost interest to identify this risk population of patients. It has been shown that the best molecular prognostic marker is mutational status of immunoglobulin heavy chain gene (IgVH). Patients whose CLL cells have unmutated IgVH genes have a

significantly worse prognosis than those whose IgVH genes have undergone somatic mutations. However, sequencing of IgVH gene is expensive and it can hardly be performed routinely in the laboratory. In order to find more convenient molecular marker, gene expression profiling has been employed to compare mutated and unmutated cells of CLL (65–67). The gene coding for ZAP-70, a tyrosine kinase protein normally expressed in T and natural killer cells, was highly expressed in unmutated cells but not in mutated cells. Subsequent studies have proved that ZAP-70 is a good surrogate marker for IgVH mutational status (68, 69).

Inherited cancer- cancer syndromes

Some familial cancers offer the opportunity to identify people at high risk to enable screening or prevention. An apparently isolated case of hemangioblastoma of the cerebellum may have occurred in someone coming from a family with von Hippel-Lindau disease. Such families are at risk of kidney cancer at the middle age and will probably benefit from screening. About 1% of all cancers arise in individuals with unmistakable hereditary cancer syndrome. Despite their rarity, inherited cancer syndromes are of great biological and clinical importance because they offer a chance to understand molecular mechanisms of cancer which leads to new approaches to the therapy.

Although, the most inherited cancer genes appear to be expressed in all adult tissues, germline mutation is often manifested only in limited number of cancers. For example, children carrying germline RB1 mutation are 90% at risk of developing one or more retinoblastomas, or osteosarcoma. These observations are puzzling and two possible explanations have been proposed: it is possible that RB1 gene is the primary controlling element in the growth regulation of retinoblast and its inactivation leads to the clonal outgrowth of precancerous cells. In other cells, RB1 may have a less critical role in growth regulation, and its inactivation may not promote neoplastic growth unless other genetic defects are also present.

Inherited colon cancer syndromes that are of the utmost interest are familial adenomatous polyposis (mutation in the adenomatous polyposis coli gene –APC gene), and hereditary non-polyposis colon cancer (HNPCC) which stems from a germline mutation of any of at least five different mismatch repair genes (MSH2, MLH1, PMS1, PMS2, MSH6). Individuals who inherit a germline mutation in an APC allele usually develop hundreds of thousands of adenomatous polyps in the colon by the age of 30, with a high risk of developing colorectal carcinoma by the age of 40. For that reason prophylactic colectomy in the early 20s is the best management for preventing development of malignant tumor.

Those who carry a germline mutation in one allele of mismatch-repair genes are at high risk of colorectal cancer (HNPCC) and in addition, increased risk of endometrial, ovarian, gastric and urinary tract cancers, as well as brain tumors and lymphomas are found in some families. Genetic counseling and testing family members

with predisposition to HNPCC can prevent unnecessary colonoscopy.

Breast cancer is the cancer with the highest incidence among women and about 5–10% of breast cancer results from genetic predisposition. Two genes that, when mutated, are associated with an increased risk of breast cancer have been identified: BRCA1 and BRCA2. Each gene encodes a large protein that may function in the cellular response to DNA damage. BRCA1 mutations, and to a lesser extent BRCA2 mutations, also increase risk for ovarian cancer. BRCA2 mutations specifically increase the risk for breast cancer in male carriers and pancreatic cancer in both sexes. Around 10% of women who develop breast cancer under the age of 40 harbor a BRCA1 mutation and 2–3% have a BRCA2 mutation, irrespective of family history. The lifetime risk for developing breast cancer in mutation carriers is 60–80%. Population that is appropriate for genetic testing includes one or more of the following: those who have three or more relatives with breast cancer; those who have two or more relatives with ovarian cancer; those who have a first-degree relative who developed breast cancer under 35 years of age or have a first-degree male relative who developed breast cancer; or conceivably, all women belonging to the ethnic group in which a few recurrent mutations can be readily identified, such as Ashkenazi Jewish women. The main dilemma with testing genetic predisposition to the breast cancer arises from the fact that even surgical management such as prophylactic bilateral mastectomy and bilateral oophorectomy, while lowering cancer risk, may not offer absolute protection from cancer. This may be partly explained by the fact that other genes carrying predisposition to breast cancer have not still been found. Furthermore, if a woman tests negative for BRCA1, she still has a standard lifetime risk of breast cancer about 10%. A negative test should not be allowed to decrease normal clinical surveillance including self-examination and mammography.

Multiple endocrine neoplasia (MEN) is characterized by the occurrence of tumors which involve two or more endocrine glands in a single patient or in close relatives. There are two types of MEN syndrome, with distinct patterns of tissue involvement. MEN1 (Werner syndrome) includes tumors of parathyroid, pituitary and pancreatic islet cells and less frequently adrenocortical, carcinoid and multiple lipomatous tumors. MEN1 is dominantly inherited. MEN2 includes tumors of the thyroid and adrenal medulla and hyperplasia or adenoma of the parathyroids. The predisposing gene for MEN2 is *ret*, a receptor or tyrosine kinase which maps to chromosome 10q11.2 and majority of MEN 2 families have detectable mutations in *ret*. The human MEN 1 and MEN 2 syndromes are genetically and almost always clinically distinct. Familial pheochromocytomas occur in two other human inherited cancer syndromes: von Hippel-Lindau syndrome and neurofibromatosis type 1. Regular screening by biochemical testing and imaging, followed by surgery when necessary, has been shown to be effective in preventing mortality and morbidity in

MEN 2 families. In a family known to have MEN 2 in which the causative mutation can be identified, DNA testing of unaffected family members at risk will eliminate those who do not have the mutation from the need for biochemical screening and simplify the decision to have surgery in those whose screening results are equivocal. Increasingly, opinion is moving toward a recommendation for thyroidectomy in childhood on the basis of DNA testing alone, without waiting for other testing showing C-cell hyperplasia (70, 71).

Molecular therapy of cancer

Cancer results from a series of mutations in oncogenes and tumor suppressor genes that disrupt controls on cell growth. This knowledge provides a rational basis for the development of entirely new avenues of anticancer therapy. The most direct approach, but currently impossible, is to fix the gene alterations in DNA through genetic engineering. Instead of that, gene therapy uses insertion of functional gene in the cells of interest. In the broader sense gene therapy can include all therapies that can modify gene expression. In that sense, cancer treatment with monoclonal antibodies (lymphoma/leukemia: anti-CD20, breast cancer: anti-her2/neu, lung cancer: anti-EGFR, anti-VEGF), specific inhibitors of protein function (inhibitor of bcr-abl tyrosine kinase activity in CML patients), antisense nucleotides and ribosimes (to: bcl-2, bcr-abl, K-ras, HPV16-E7, c-myc, myb) also can be considered as gene therapy (72, 73).

Gene therapy can be defined as the transfer of genetic material with therapeutic intent. Although inherited genetic diseases are an obvious target for gene therapy approaches, most gene-transfer clinical protocols submitted to date have involved patients with cancer. The methods used to transfer nucleic acids (oligonucleotides, DNA or RNA) into mammalian cells, either *ex vivo* or *in vivo*, form the basis of all approaches to gene therapy. The current methods used for gene transfer into mammalian cells can be described in two broad categories, physical-chemical or viral. Examples of the physical-chemical approach include direct introduction of the DNA using injection, particle bombardment, or electroporation, calcium phosphate transfection, complexation with cationic lipids or polymer, or linkage to a specific ligand, and the use of corresponding receptor. Viral vectors have been developed from many different viruses, from small RNA-based viruses to complex DNA viruses. Viral vectors are the most efficient vehicles for gene delivery because these make use of receptors or other interactions with the cell. Current gene-delivery systems all have advantages and limitations for the treatment of cancer, but retroviral vectors are mostly exploited for gene therapy. An ideal vector should: (a) allow efficient and selective transduction of the target cell of interest; (b) be maintained; (c) be expressed at levels necessary for achieving therapeutic effects, and (d) be safe in terms of avoiding unexpected side effects in the host.

A number of approaches have been adopted to exploit the potential of cancer gene therapy: restoration of phe-

notype; enhancement of cytotoxicity; cytoprotection and immunomodulation.

One of the method by which malignant phenotype could be reversed is by inhibition of oncogene expression. This can be achieved by inhibition of oncogene transcription or translation. Nucleotide sequences complementary to the relevant mRNA (antisense oligonucleotides) prevent translation and promote mRNA degradation. Vectors which encode antisense oligonucleotides capable of blocking oncogene mRNA have been shown to be feasible in different preclinical studies (74–77).

Restoration of tumor suppressor gene function in tumor cells *in vitro*, by transduction with wild-type gene, causes reversion of malignant phenotype and often apoptosis (78, 79). Replacement of p53 gene and restoration of function were demonstrated in non-small cell lung cancer, breast, prostate and head and neck tumors using adenoviral vectors that selectively infect p53 deficient cells (ONYX-015).

Some gene therapy strategies are directed at enhancing the selectivity of cytotoxic therapy or restoring tumor cell sensitivity to cytotoxic agents. Suicide gene therapy is a two-step treatment for solid tumors. In the first step, the aim is to deliver a gene for a foreign enzyme to the tumor in a vector. Following expression of the foreign enzyme, a prodrug is administered in the second step, which is activated into the drug selectively in the tumor. The enzyme should be expressed exclusively in the tumor cells, or with relatively high ratio compared to normal tissues and blood. The enzymes proposed for suicide gene therapy include: herpes simplex virus thymidine kinase (HSV-TK), bacterial enzymes such as cytosine deaminase (CD), purine nucleotide phosphorylase (PNP), carboxypeptidase G2 (CPG2) and nitroreductase bG2 (NR). Unlike mammalian TK, HSV-TK preferentially monophosphorylates ganciclovir into toxic metabolites and induces cell death. In this approach it is not needed that all tumor cells be transduced with HSV-TK gene, since toxic metabolites may be transferred through gap junctions to neighboring cells („bystander effect“). The first successful application of this therapy was treatment of glioblastoma multiforme (80).

Other enzyme/prodrug combinations being investigated include bacterial cytosine deaminase, which converts relatively non-toxic 5-fluorocytosine to 5-fluorouracil (colon and liver cancer).

Reducing sensitivity of stem cells in bone marrow to cytotoxic agents could increase tolerance to higher doses of chemotherapy. In preclinical studies, using retroviral vectors to transfer human MDR1 (multidrug resistance gene), glutathione-S-transferase and dihydrofolate reductase, different groups have shown that this approach is possible (81).

The majority of clinical trials utilizing gene therapy have had the goal of inducing or augmenting immunologically mediated tumor rejection. Strategies to augment the immune response to tumor have included attempts to increase the immunogenicity of tumor cells. Tumor cells transfected with cytokine genes, such as IL-2,

IL-12, GM-CSF, can induce a greater antitumor response and have caused regression in animal models. Whilst some groups have utilized modified tumor cells, others have focused on modification of immune cells, such as dendritic cells. The aim of this strategy is to harness the endogenous antigen-presenting machinery of the dendritic cell and allow it to identify epitopes within tumor-associated antigens that bind to host MHC. These epitopes may then be presented to T cells to induce a specific immune response.

Parenteral administration of viral vectors expressing tumor-associated antigens can result in specific-cell mediated immunity and regression of tumor. It is also possible to engineer vectors that express costimulatory molecules or cytokines as well as tumor-associated antigens.

Naked plasmid DNA encoding tumor-associated antigens have also been used as a gene therapy. Both methylation and high proportion of CpG motifs within bacterial DNA may stimulate a local inflammatory infiltrate.

Clinical cancer gene therapy trials reported so far have failed to show significant therapeutic efficacy partly because most of them recruited patients with advanced, drug-refractory disease. An important problem causing unsatisfactory clinical results is the poor targeting selectivity of the used vectors, leading to low efficiency of gene transfer to tumor cell. Although the search for new vectors (viral and non-viral) and more selective targeting moieties will continue, meanwhile it is worthwhile to

take seriously into consideration using of combination therapy, i.e. simultaneous administration of conventional chemotherapy with different gene therapy approaches (82-84).

CONCLUSION

There is a hope that the time will come soon when oncologists will be able to characterize the molecular fingerprint of a tumor, which represents the genetic profile of tumor. Such molecular fingerprint might then provide clues to help to determine whether a tumor will be fast or slow growing, whether it will metastasize, and if it will respond to particular treatment. Although these are mostly expectations at the moment, many clinical studies of clinical significance of various genetic alterations are guarantees that these tests will soon become part of clinical reality. Molecular targets for diagnosing and treating cancer, molecular staging of cancer, individualization of therapy, inherited cancer and gene therapy are some of the most promising areas of clinical molecular oncology. To achieve these targets it is necessary to improve knowledge in human genetics through education in medical school and constant after-graduate education. Also, it is of the utmost interest for the society to finance scientific projects for the cancer research and to introduce new technologies into everyday oncological practice.

Professor dr Zvonko Magić

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