

## Use of molecular biology techniques in cancer epidemiology

by Kari Hemminki, MD<sup>1,2</sup>

HEMMINKI K. Use of molecular biology techniques in cancer epidemiology. *Scand J Work Environ Health* 1992;18 Suppl 1:38-45. Biochemical techniques have been applied for years in clinical medicine to unravel disease indicators and mechanisms. The study design, resembling that of a case-referent study, has been to compare diseased and healthy individuals. Conclusive results have been obtained for diseases in which relatively few factors cause high risks. The same study design has been maintained with sophisticated methods of molecular biology, which have revealed many types of deoxyribonucleic acid lesions associated with cancer. It is likely that the relatively simple cross-sectional and case-referent studies require input from epidemiologists to unravel multifactorial etiologies. Longitudinal approaches may be the only alternative with which to evaluate the role and usefulness of the application of molecular biology markers in studies of cancer.

**Key terms:** constitutional factors, deoxyribonucleic acid adducts, genetic factors, molecular epidemiology, mutations, oncogenes, protein adducts.

Etiology-oriented epidemiologic research is involved in the study of disease frequency with the objective of establishing causes of diseases (1, 2). It has been successful in many areas. For example, many risk factors for cardiovascular diseases and carcinogenic agents have been established which have helped in the design of preventive measures. However, even modern epidemiologic methods have limitations when multiple etiologic factors and small risk ratios are involved. Furthermore, epidemiologic findings rarely establish the mechanism of the disease. This problem has led to introspection among epidemiologists and to discussions about the exploitation of molecular biology for epidemiologic studies (3, 4).

Molecular biology<sup>3</sup> seeks to understand the biological basis of life. It frequently addresses the differences between normal and diseased persons or cells and thereby attempts to unravel disease mechanisms. Major advances have recently been made in this field, including insight into cellular function and regulation. This development has helped to identify genes and enzymes that play an important role in many diseases. The ultimate sequencing of the total human genome (HUGO) has been launched, and, once completed, the genes of all the proteins and regulatory sequences will

be known. This information will provide an immense base for hypothesis testing in disease etiology.

Over the years epidemiology and biochemical research have conceptually fed on each other. Epidemiologic research, identifying causes and risk factors, has provided biochemists with clues to study, and visa versa. However, there has been relatively limited interplay between biochemists and epidemiologists. Examples of studies in which biochemical parameters have been extensively used in refined epidemiologic studies include the ones in which blood lipids, vitamins, and microelements have been correlated to risks of cardiovascular disease and cancer. More recently epidemiologic study designs have been exploited in molecular biological studies. Such studies have a unique possibility to unravel disease mechanisms at the gene level (5, 6). Optimally, the combination of molecular biological and epidemiologic research (molecular epidemiology) can establish biological disease indicators, markers, which could substitute for actual epidemiologic studies.

Such hopes have been crystallized in the term molecular epidemiology, coined by Perera & Weinstein (7). According to Perera, "molecular epidemiology seeks to combine the precision of laboratory methods to quantify carcinogenic dose or preclinical response in humans with the relevance and vigor of analytic epidemiology [p 887]" (8). She continues, "just as infectious disease epidemiology was greatly advanced by methods to identify viruses and to elucidate the mechanisms involved, biologic markers of dose and response spring from greater understanding of basic biologic processes and have significant potential in identifying and assessing carcinogenic risks to humans from exposures to man-made environmental chemicals [p 887]." Molecular biology can contribute to epidemiologic research by providing measures of exposure, effect, and susceptibility to disease.

<sup>1</sup> Institute of Occupational Health, Helsinki, Finland.

<sup>2</sup> Center for Nutrition and Toxicology, Karolinska Institute, Novum, Huddinge, Sweden.

<sup>3</sup> Molecular biology is defined here as the study of the structure and function of the genes and their macromolecular products. Biochemistry is a wider discipline, encompassing molecular biology. However, in this context, biochemical research refers to the study of small molecules, enzyme activities, and metabolic parameters.

## Previous applications of biochemical epidemiology

Epidemiology pertains to the observation of disease frequency in respect to various characteristics of the population. Thus medical textbooks abound with epidemiologic observations (ie, various correlates or risk factors of diseases). The area of medicine in which biochemistry and epidemiology have had a traditional, fruitful interaction is medical genetics. Hundreds of genetic and metabolic diseases have been characterized, and essential concepts have been established. These concepts could serve as a paradigm for a later discussion in this paper. It would thus be instructive to analyze why the marriage of epidemiology and biochemistry has been so successful in medical genetics (9, 10).

One undoubted reason for the success has been the foundations of genetics established since the days of Mendel. Another reason is that the penetration of some genetic diseases has been so high that they have not escaped clinical observation, and it has been easy to establish family pedigrees. A third reason is the clear deficiency of a normal enzyme in monogenic forms of a disease. The symptoms of disease have guided scientists to search for certain types of enzymes. Fourth, many genetic diseases are manifested through severe chromosomal changes detectable even with early karyotyping methods.

By the same token one might ask why biochemical epidemiology has hardly had any success in some other areas of medicine, such as psychiatry. According to the textbooks, the heritability of, for example, schizophrenia is high, and therefore it should be as equally amenable to biochemical and epidemiologic analyses as some of the typical diseases of medical genetics. Yet neither the biochemistry nor the epidemiology of schizophrenia has provided many clues to its etiology. The reasons may lay in the multigene origin and a complex interplay between genetic and environmental factors.

## Genes and environment

Modern medicine is based both in study and in practice on a profound understanding of cellular function and the interplay between various organ systems. In short, the understanding is that one set of chromosomes is inherited from the maternal and the other from the paternal side. Genes in chromosomes code for proteins that are enzymes and structural components. Enzymes metabolize nucleic acids, lipids, carbohydrates, and other proteins. The whole system is regulated by a complex network of nutrients, hormones, and growth factors through feedback mechanisms.

These elementary facts are presented because they are the limits of consensus in medical thinking. Very diverse opinions are presented on the role of genetic and environmental factors in disease etiology. These

are partially terminological differences. Distinction should be made between inheritance through germ cells (hereditary) and genetic constitution (combination of hereditary and somatic events). It is now well understood that the inherited deoxyribonucleic acid (DNA) is under continuous change through recombinations and mutations, which may be endogenous or environmentally caused. Some of the confusion in clinical genetics and other fields of medicine is caused by the term "heritability" defined as "the proportion of the total variation of a character attributable to genetic as opposed to environmental factors [p 115]" (9). This term considers environmental and genetic events distinct and fails to observe the influence of environmental factors on genes.

One has to be cautious in using the terms "genetic" and "environmental," and one must not confuse the terms "genetic" and "hereditary." Medical texts frequently refer to the fraction of a disease attributable to genetic and environmental factors. Such figures may be of some practical use in simplifying disease etiology, but due attention should be paid to the definition of the terms.

When diseases have multiple etiologies, as almost all do, attributable causes do not have to add up to 100%. For this reason it has been pointed out somewhat polemically that almost any disease can either be shown to be environmental or genetic in origin (2).

For medical practice hereditary diseases are important for genetic counseling, whereby disease risk may be lowered in the next generation. In addition many hereditary disorders are associated with special sensitivities to environmental agents, and diseases may be alleviated or prevented by intervention (11, 12). When a disease is caused by environmental factors, primary prevention is possible. For this reason much of analytic epidemiology has been directed towards finding these factors.

The interplay of hereditary and environmental factors is illustrated in figure 1. Environmental factors may have caused mutations in the germ cells of the previous generation, which either manifest themselves as hereditary diseases or increased susceptibility to disease in the progeny. In the progeny environmental factors can affect DNA directly, causing mutations, or epigenetically, changing the gene expression in a harmful way and making the individuals more susceptible to disease.

Most of the events illustrated in figure 1 are well documented in medical literature. The knowledge on DNA lesions involved in hereditary diseases is growing fast. One example is glucose-6-phosphate dehydrogenase deficiency that predisposes the patients to drug-induced hemolysis (11). Even though the DNA lesion is characterized, its mechanism of formation (whether an endogenous or exogenous event or both) is not known.

It holds true for many of the common diseases affecting progeny that mutations are involved. The part

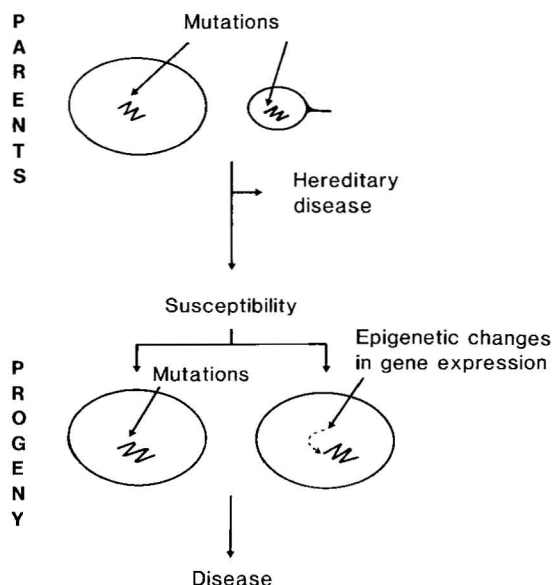


**Figure 1.** Effects of environmental factors on parental germ cells and somatic cells of progeny.

of figure 1 which is least documented is the epigenetic change of gene expression by environmental agents. The induction of drug metabolizing enzymes by drugs and environmental chemicals, as discussed later, changes in enzyme activities in occupational poisonings, and changes in immune response due to exposure to dioxins may be examples of such mechanisms. In addition various allergic conditions are perhaps examples of epigenetic disease mechanisms caused by environmental agents.

The heritability of a disease is analyzed through the construction of family pedigrees. The demonstration of familial clustering is, however, a relatively insensitive indicator of genetic predisposition. Peto (13) has shown that even if the risk in probands is high, the incidence of a disease in relatives is only moderately increased. The availability of molecular markers for the disease would make the analysis more sensitive.

### Molecular epidemiology of cancer

Cancer is thought to be a multistage process in which cells lose their normal growth control through a number of successive stages (14–16). The ideas of a multistage process arose mainly from epidemiologic analyses of age dependence and latency periods in the onset of cancer (17–20). The early observations of malignant growth in *in vitro* systems led Boveri (21) to propose that permanent changes underlie malignant growth. It was noted later for human populations that many cancers have a hereditary component (22–23). Knudson & Strong (24) analyzed the heredity of retinoblastoma, Wilms' tumor, neuroblastoma, and pheochromocytoma and postulated mutational eti-

ology. In the case of retinoblastoma it was noted that the bilateral and unilateral forms of the disease differed in heritability, age of onset, and number of tumor clones. It was supposed that the bilateral form required one mutation, whereas the unilateral form required two mutations in the same loci. It was reasoned that, in the bilateral case, a mutated allele in the retinoblastoma locus was inherited and thus only one somatic mutation was required (25). Such epidemiologic analyses have recently been confirmed by molecular genetics and have led to a marked advancement in the understanding of the genetic bases of certain cancers and the refinement of the critical genes, oncogenes, and antioncogenes interplaying in malignant transformation (14, 26).

Parallel to the studies on the genetic basis of cancer other lines of cancer research were also proliferative and pointed in the same direction as genetic studies. Extensive work with chemically induced cancers have led to an overall understanding that a large proportion of chemical carcinogens are electrophiles, able to react covalently with DNA (27). These chemicals are often mutagenic (28). When the genotoxicity of well-documented human and animal carcinogens have been summarized, an overwhelming proportion, 80–90%, have been found to be positive in various genotoxicity assays (29). Taken together, these data indicate that DNA is the target for many carcinogens, and their mechanism of action is to cause a somatic mutation in some of the critical genes, as has already been outlined.

A third line of cancer research used epidemiologic data on the incidence of cancer in various countries and in immigrant populations to conclude that the causes of cancer are mainly environmental (30, 31). This conclusion excludes dominant inheritance as being the cause of the most common cancers, but it does not exclude the role of somatic mutations.

A discussion of the multistage process, in which constitutional factors and environmental exposures interact, follows, whereas early diagnostic measures are outside the scope of this survey.

### Constitutional factors

Several constitutional factors underly susceptibility to cancer. These include, among others, levels of enzymes that activate and inactivate chemical carcinogens and levels of DNA repair enzymes (32, 33). The activities of several of these enzymes have been studied in human tissue explants *in vitro*, and marked interindividual differences, up to several 100-fold, have been noted (table 1). To what extent these polymorphisms in human populations actually predispose individuals to cancer has been a field of active research, and some suggested relationships are presented in table 2. It has not been established that the carcinogens indicated in the table are the sole factors. Furthermore many controversial data exist. In a recent study persons who ex-

tensively metabolized debrisoquine were found to be at a fourfold risk of lung cancer when the data were adjusted for age, gender, and smoking. As comparison, in the same study, occupational exposure of men to asbestos and polycyclic aromatic hydrocarbons (PAH) carried a 2.9- and 2.4-fold risk, respectively (34).

Some genetic diseases are known to carry an increased risk of cancer (table 3). Among these, xeroderma pigmentosum has defects in DNA repair, and in ataxia-telangiectasia and Fanconi's anemia somatic chromosome aberrations are frequently found in the patients. Down's syndrome and polyposis coli are associated with an increased risk of leukemia and colon carcinoma, respectively, but the molecular mechanisms have not been unraveled. In the hereditary form of retinoblastoma there is a mutation in one allele of band q in chromosome 13 (14).

#### Measurement of exposure

Direct measurement of a chemical or its metabolite in ambient air or in biological fluids is the most common

means of assessing exposure in epidemiologic studies. However, usually concurrent, representative individual measurements are lacking, and exposure categories are constructed from sparse data combined with other hygienic knowledge.

Recently methods have been developed to measure the covalent binding products of carcinogens with DNA and protein. The background for these methods is that the binding of a chemical to DNA is thought to be the initiating event triggering the cascade of biochemical changes eventually leading to malignancy (15). In target tissues of experimental animals the levels of adducts correlate with tumor responses (35, 36). This phenomenon has not been directly studied in humans, but inferences can be made from animal studies and from the indirect data, discussed later, on groups at high risk of cancer. Because target tissues are usually not available for humans and the DNA binding studies resort to surrogate tissue, the aforementioned inference is less compelling. Generally, binding to protein, such as hemoglobin and albumin, correlates with binding to DNA, and protein adducts can be used as a surrogate measure of DNA binding (37, 38).

**Table 1.** Interindividual variation in carcinogen-metabolizing and DNA-repairing enzymes in human tissues.<sup>a</sup> (DNA = deoxyribonucleic acid)

| Enzymes                           | Tissues studied                             | Variation reported |
|-----------------------------------|---------------------------------------------|--------------------|
| <i>Carcinogen metabolism</i>      |                                             |                    |
| Aryl hydrocarbon hydroxylase      | Liver, lung, placenta, skin, kidney         | 7—350              |
| Epoxide hydrolase                 | Liver, lung, placenta, skin                 | 3—500              |
| Glutathione transferase           | Liver, lung, heart, brain, pancreas, rectum | 2—50               |
| <i>DNA repair</i>                 |                                             |                    |
| O <sup>6</sup> -methyltransferase | Liver, colon, small intestine               | 8—40               |
| Uracil DNA-glycosylase            | Liver, stomach, small intestine, colon      | 3—65               |

<sup>a</sup> Modified from Vähäkangas & Pelkonen (32).

**Table 2.** Suggested relationships between xenobiotic metabolism and risk of cancer.<sup>a</sup>

| Metabolism                             | Xenobiotics suspected                          | Relationship                                |
|----------------------------------------|------------------------------------------------|---------------------------------------------|
| Acetylation                            | Aromatic amines                                | Bladder cancer risk with slow acetylation   |
| Debrisoquin hydroxylation              | Polycyclic aromatic hydrocarbons, nitrosamines | Lung cancer risk with extensive metabolism  |
|                                        | Aflatoxin B <sub>1</sub>                       | Liver cancer risk with extensive metabolism |
| Aryl hydrocarbon hydroxylase induction | Polycyclic aromatic hydrocarbons               | Lung cancer risk with high induction        |
| Glutathione transferase                | Many carcinogens                               | Lung cancer risk with low activity          |

<sup>a</sup> Modified from Vähäkangas & Pelkonen (32).

**Table 3.** Syndromes and diseases with a high risk of cancer.<sup>a</sup>

| Syndrome              | Frequency                                     | Type of neoplasia |
|-----------------------|-----------------------------------------------|-------------------|
| Xeroderma pigmentosum | 1/250 000 homozygotes, 2—4/1000 heterozygotes | Skin cancer       |
| Ataxia telangiectasia | 2.5/100 000 homozygotes, 1/100 heterozygotes  | Lymphoreticuloses |
| Bloom syndrome        | ..                                            | Leukemia          |
| Fanconi's anemia      | 1/1 000 000 homozygotes, 1/300 heterozygotes  | Leukemia          |
| Down's syndrome       | 1/1000                                        | Leukemia          |
| Polyposis coli        | ..                                            | Colon cancer      |
| Retinoblastoma        | ..                                            | Retinoblastoma    |

<sup>a</sup> Main source Calabrese (11, 12).

The development of sensitive methods based on  $^{32}\text{P}$ -postlabeling (39–41) or immunoassay (42) have laid the groundwork for the determination of DNA adducts in humans (figure 2). The technology is new and ever improving and expanding (43). Due caution has to be exercised in the interpretation of the data obtained as to the accuracy and identity of the measurements. Recently interlaboratory comparisons have been carried out either for the postlabeling technique, immunoassay, or both, and reasonable correlations have been found (44). Yet unknown variables exist, and there is no proof that in human DNA the two assays measure identical adducts.

The two techniques have been applied in occupational and environmental studies (45, 46). One population has been foundry workers that are known to be at risk of lung cancer (47). In a series of studies it has been shown that the levels of aromatic adducts in white blood cell DNA of workers correlate with the estimated exposure to PAH (44, 48, 49).

#### Measurement of effects

Cytogenetic end points, chromosome aberrations, sister chromatid exchanges, and micronuclei have been used extensively in occupational monitoring (50). In addition to some 10–20 occupational exposures several anticancer agents have increased one or several of the cytogenetic end points in humans (29). Many of these exposures also increase the risk of cancer. At the group level an increase in chromosome aberrations is usually associated with a risk of cancer (51). This result is also supported by the increased incidence of cancer in the syndromes which involve chromosome instability, such as ataxia-telangiectasia and Fanconi's anemia. (See table 3.) In the Nordic countries a collaborative follow-up study has recently been initiated

in which cytogenetic data on the frequency of chromosome aberrations, sister chromatid exchanges, and micronuclei are being correlated with the risk of all cancers (52).

Karyotyping of cancer cells has revealed many consistent changes. By now more than 10 000 abnormal karyotypes have been cataloged (53), and many are related to a risk of cancer. The first of these changes was the translocation between chromosomes 22 and 9 (the Philadelphia chromosome) present in some 98% of the patients with chronic myeloid leukemia (53). This translocation is sometimes observed before the clinical disease, which however appears with about 95% probability. It has been shown by molecular probes that the translocation involves the transfer of the *abl* oncogene to chromosome 22 (54).

Karyotype analysis of chromosome deletions and translocations has led to molecular mapping methods that can be applied to retinoblastoma and chronic myeloid leukemia, as has already been discussed. Analysis for restriction fragment length polymorphism (RFLP) involves digestion of DNA with restriction enzymes, gel electrophoresis for separation by size, and Southern hybridization using radioactive probes towards defined loci in DNA. With this technique changes can be seen in the length of the allelic DNA sequences indicative of deletions, insertions, or other rearrangements. After the work with retinoblastoma, losses of the normal allele (ie, loss of heterozygosity) have been observed for several human tumors (14).

Techniques for the molecular analysis of various types of DNA lesions or of aberrant gene expression are listed in table 4. While Southern blot techniques (RFLP) are best suited to detect large lesions in DNA, point mutations can be scored by polymer chain reaction (PCR), in which the sequence of interest is amplified thousands of times (55). Polymer chain reaction can be run after denaturing gel electrophoresis if rare mutants are detected among normal cells. This procedure has been applied with human hypoxanthine guanine phosphoribosyltransferase (HPRT) genes (56). Ionizing radiation (57) and anticancer agents (58) have been shown to cause mutations in the HPRT gene in humans. In the analyses of tumors gradient gel electrophoresis is not needed because the cells are clonal and a polymer chain reaction can be carried out with total DNA (59). This procedure has been applied to point mutations in the *ras* gene.

Two new techniques are used to assay for proteins. In the glycophorin A assay antibodies specific to the M and N alleles are used with flow cytometry and cell sorting. The variants lacking one of the alleles are isolated, and the presumed mutants are scored. The frequency of variants is about  $1 \cdot 10^{-5}$  in normal blood donors and increases in individuals exposed to anticancer agents and radiation (60). The other protein assay measures the amount and size of oncoproteins by immunoblotting (61). *Ras* and *fes* oncoproteins are detected in plasma in some forms of cancer (62), and even

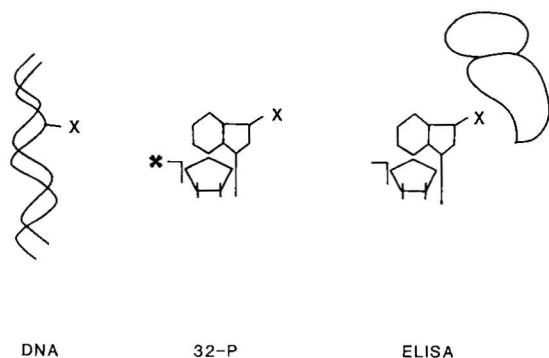


**Figure 2.** Methods for the measurement of deoxyribonucleic acid (DNA) adducts in humans. Left: an adducted DNA; middle: a  $^{32}\text{P}$ -postlabeling method in which polynucleotide kinase introduces a high-specific activity phosphate group to the 5'-position; right: antibody detection of a modified nucleotide. (X = carcinogen, ELISA = enzyme-linked immunosorbent assay)

**Table 4.** Molecular analysis of mutations and gene expression in relation to cancer. (RFLP = restriction fragment length polymorphism, PCR = polymer chain reaction, HPRT = hypoxanthine guanine phosphoribosyltransferase)

| Assay                                  | Applications                                                                              |
|----------------------------------------|-------------------------------------------------------------------------------------------|
| RFLP                                   | Differences in allele size, number, loss of heterozygosity, probing of antioncogenes      |
| Southern hybridization                 | Amplification of oncogenes, large deletions/rearrangements                                |
| PCR±denaturing gels                    | Probing of point mutations, <i>ras</i> , HPRT                                             |
| Glycophorin A antibodies, cell sorting | Identification of variant erythrocytes lacking one of the M or N alleles in heterozygotes |
| Western immunoblotting                 | Analysis of oncoproteins, expression and type                                             |

in some occupational groups exposed to polychlorinated biphenyls (63) and PAH (64).

### Aspirations in molecular epidemiology

Molecular biological and epidemiologic studies have benefited each other conceptually in forming research hypotheses. In practical science the interaction has been meager. In cancer studies blood determinations of body constituents have been carried out, but they have been simple chemical determinations of vitamins and micronutrients. On the other hand the design of a typical molecular biology study in medicine has been to assay samples from diseased and healthy individuals. This procedure has lead to conclusive results when the factors under study have caused large risks. For smaller risks the formal case-referent approach using consulting epidemiologists could be necessary.

Molecular biology using specimens from diseased and healthy individuals obviously continues on its own merit, but such an approach does not combine the power of molecular biology and epidemiology in the form of molecular epidemiology. Molecular epidemiology can reveal constitutional exposure or effect indicators that predict the disease with a high probability. Many such indicators or markers have been listed in this presentation. The predictivity of the risk factor can only be tested epidemiologically. If high predictivity markers are found, they will serve the purpose of disease prevention or intervention to delay the appearance of the disease.

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