Somatic and Germinal Mutations of Tumor-Suppressor Genes in the Development of Cancer

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It is generally thought that the germinal mutation of tumor-suppressor genes predisposes the affected children to the development of certain types of hereditary tumors while the somatic mutation of the same genes links to the development of non-hereditary tumors. Retinoblastoma susceptibility gene (RB gene) is a prototype of such genes. We studied the parental origin of new mutation of the RB gene in the sporadic hereditary and non-hereditary retinoblastoma and osteosarcoma. The results showed a preferential involvement of parental genome in the new germinal as well as initial somatic mutations. The male-directed mutagenesis even in the somatic cells has been implicated as a reflection of germinal origin of mutation, even for non-hereditary tumors as a manifestation of mutational mosaicism associated with delayed mutation. The importance of the new mutations occurring as mosaics should be emphasized in the evaluation of cancer risks from parental exposures to radiation and chemicals.

INTRODUCTION

Evidence has been accumulated for the involvement of the loss-of-function mutation of the negative regulatory genes, *i.e.*, tumor-suppressor genes, in the development of a variety of human cancers, particularly solid tumors^{1,2}. Retinoblastoma susceptibility gene (RB gene) is a prototype of such genes. Its loss-of-function mutation predisposes to the development of retinoblastoma and osteosarcoma. The mutations are either germinal or somatic; the germinal mutation involving RB gene leading to the development of hereditary tumors while the somatic mutation of the same gene is manifested as non-hereditary tumors.

In view of an increasing concern about the parental origin of cancers in radiation- and chemical protection standard^{3,4}, the origin of mutation of such tumor-suppressor genes are

particularly intriguing. In this paper, we presented our recent observations on the parental origin of mutations of such tumor-suppressor genes, and discussed their possible implication in the variabilities of the development of cancers, particularly childhood cancers. The observations point to the strong bias toward paternal origin. Male-derived mutation could be postulated, as a testable proposition, for the development of childhood cancers including so-called non-hereditary cancers as a variegated mutational mosaicism.

MATERIALS AND METHODS

Retinoblastoma patients who were referred to the National Cancer Center Hospital, Tokyo, were studied for the presence of constitutional chromosome aberrations in their cultured skin fibroblasts as described⁵⁾. Genomic DNA was also purified from the cultured skin fibroblasts and tumor tissues according to standard methods⁶⁾, and studied by Southern blot analysis for the abnormalities of RB gene using cDNA probes of RB gene and changes of the constitutional organization of chromosome 13 using polymorphic DNA probes which reveal RFLP in the genomic DNA when digested with appropriate restriction enzymes. When available, the somatic cells, either cultured skin fibroblasts or peripheral blood leukocytes, were also subjected to the analysis in order to identify the parental origin of mutation.

Similar analysis has been done for the patients with osteosarcoma and their tumors as described⁷⁾.

RESULTS AND DISCUSSION

Parental Origin of Germinal Mutation

In our consecutive survey of 254 retinoblastoma patients, 14 showed the constitutional chromosome abnormalities which could be recognized under the microscope. The abnormalities were all *de novo* origin and were interstitial deletions involving 13q14 in 10 cases, autosomal reciprocal translocation with a breakpoint at 13q14 in 3 cases and X/13 translocation in 1 case. The X/13 translocation was between chromosome 13 and inactive X chromosome, and assumed to be the case of transcriptional shut-off of the RB gene due to the spreading of X chromosome inactivation⁸⁾. The parental origin of these chromosome mutations were studied by the use of quinacrine fluorescence pattern of 13p as heritable markers. In 10 out of 11 informative cases, the chromosome mutations were paternal origin. Recently, Huff *et al.* (1990)⁹⁾ also reported that constitutional deletions involving 11p13 in patients with aniridia-Wilms tumor association were paternal origin in 7 out of 8 cases.

According to the two mutation paradigm of Knudson (1971)¹⁰⁾, a single RB gene mutation is not sufficient and a subsequent loss-of-function mutation of the normal allele is needed to be invoked. This second event often involves the loss of substantial amount of homologous chromosome region by gross deletion or mitotic recombination and hence

recognized as a loss of constitutional heterozygosity in tumor cells (Fig. 1). Therefore, the retained chromosome or chromosome segment is assumed to have the initial mutation, and hence provide an opportunity to identify the parental origin of germinal mutations of subvisible type (Fig. 2). With this strategy, the genomic changes in tumors were studied in sporadic bilateral retinoblastoma and compared with the constitutional organization in the somatic cells of the patients and their parents. In all of the three informative cases, retained chromosomes were paternally derived ones, indicating the paternal origin of germinal mutation of RB gene.

The strong bias toward paternal origin for the germinal mutation of RB gene has been also reported by Dryja *et al.* (1989)¹¹ and Zhu *et al.* (1989)¹². Recently, Jadayel *et al.* (1990)¹³ also reported that the fresh germinal mutation of gene for von Ricklinghausen neurofibromatosis (NF-1) occurred preferentially in the paternal germ cells (Table 1).

Origin of Initial Somatic Mutation of Tumor Suppressor Genes

Non-hereditary tumors are assumed to arise as a consequence of two successive somatic mutations of the tumor-suppressor gene. According to the underlying mechanisms involved in the loss of heterozygosity, it can be also reasoned that the allele remaining in the tumor tags the chromosome which harbors the initial somatic mutation.

Fig. 1. Mutation of RB gene and the chromosomal mechanism of loss of heterozygosity in chromosome 13.

Fig. 2. Determination of parental origin of a new mutation of RB gene in retinoblastoma patients. (A) Familial case. (B) Sporadic bilateral case. (C) Sporadic unilateral case. High molecular weight DNA was isolated from tumors (T) and cultured skin fibroblasts of the patients and their parents. The Southern analyses were performed using indicated restriction enzymes and probes. The paternally derived allele remains in tumor in (A) and (B). The maternally derived allele remains in tumor in (C).

Table 1. Parental Origin of Germinal Mutation in the Development of Hereditary Tumors

	Mutated or retained chrom.		References
	Paternal	Maternal	
Retinoblastoma	10	1	Ejima <i>et al.</i> (1988)
	8	0	Dryja <i>et al.</i> (1989)
	4	1	Zhu <i>et al.</i> (1989)
	3	0	This paper
	Total	25	2
Aniridia-Wilms tumor	1	0	Glaser <i>et al.</i> (1989)
	7	1	Huff <i>et al.</i> (1990)
	Total	8	1
von Recklinghausen disease (NF-1)	12	2	Jadayel <i>et al.</i> (1990)

With these methods, homologue that suffered the initial somatic mutation has been identified in sporadic non-hereditary form of Wilms tumors¹⁴⁻¹⁷, osteosarcoma¹⁸, and rhabdomyosarcoma¹⁹. In the Wilms tumor and rhabdomyosarcoma, the reduction from heterozygosity to homo- or hemizyosity has been studied on the polymorphic loci on chromosome 11, more particularly on 11p region, which harbors the putative tumor-suppressor genes related with the development of these tumors. The loss of heterozygosity on chromosome 13 has been analyzed in osteosarcoma in which RB gene was thought to

be responsible for their development^{7,20-22}). As seen in Table 2, it is evident that the alleles remaining in the tumors are paternally derived ones in most of the cases. In these tumors, the non-randomness is obvious ($p < 10^{-6}$).

In contrast, in the sporadic unilateral retinoblastoma, most of which were thought to be non-hereditary and arise as a consequence of two somatic mutations of the RB gene, Dryja *et al.* (1989)¹¹ and Zhu *et al.* (1989)¹² reported an equal contribution of paternally and maternally derived copies of chromosome 13 and concluded no preferential involvement in the initial somatic mutation (Table 2).

However, these conclusion should be recriticised, since our observations showed that the loss of heterozygosity was much more common in the non-hereditary sporadic unilateral retinoblastomas than in the hereditary (bilateral or familial) tumors (Kato *et al.* unpublished results). In the consecutive survey on the genomic changes in tumors, the loss of heterozygosity was found in about 50% of the hereditary and 70% of the non-hereditary tumors. The excess of the loss of heterozygosity in the non-hereditary tumors were statistically significant and was assumed to be a reflection of the gross deletion as the initial somatic mutations (see Fig. 1d). This is supported by the facts that (1) the gross deletion involving RB gene is rare in the germinal mutations^{23,24} and hence the loss of heterozygosity is mainly due to the second events which unmask the initial events (see Fig. 1b,c), (2) unlike osteosarcoma, homozygosity of a total deletion of a region involving the RB gene is infrequent in retinoblastoma and hence the loss of heterozygosity could be the consequence of the initial somatic mutation in substantial number of non-hereditary tumors.

Table 2. Parental Origin of Chromosomes Involved in the Initial Somatic Mutation in Non-hereditary Tumors

	Mutated or retained chrom.		References
	Paternal	Maternal	
Wilms tumor	2	0	Reeve <i>et al.</i> (1984)
	5	0	Schroeder <i>et al.</i> (1987)
	4	1	Mannens <i>et al.</i> (1988)
	3	0	Williams <i>et al.</i> (1989)
Total	14	1	
Osteosarcoma	12	1	Toguchida <i>et al.</i> (1989)
Rhabdomyosarcoma	6	0	Scrabble <i>et al.</i> (1989)
Retinoblastoma	3	4	Dryja <i>et al.</i> (1989)
	1	2	Zhu <i>et al.</i> (1989)
Total	4	6	

This implies that in non-hereditary retinoblastoma the alleles remaining in the tumor do not necessarily tag the chromosome that suffered the initial mutation. Indeed, we found the loss of paternally derived alleles in 5 out of 7 non-hereditary tumors (Kato *et al.* unpublished results). When such different spectrum of the types of mutation between hereditary and non-hereditary retinoblastoma is concerned, the parental origin of the lost allele strongly points to the possibility that the non-hereditary retinoblastoma is not an exception and that the initial somatic mutation also occurs preferentially on the paternally derived chromosome.

Stage at Risk for Germinal Mutation

Strong bias toward paternal origin of the germinal mutation of the tumor-suppressor genes involved in the development of childhood cancers are interesting. An argument may be possible as to the differential germinal imprinting for the expression of the paternal and maternal alleles of the RB genes so that the loss of function of paternal allele will be more efficient in inducing tumor²⁵). To test this hypothesis, we compared the expressivity in two groups of the carrier children in the families with retinoblastoma including those in the literatures; one with mutated RB gene transmitted from father and the other transmitted from mother. The results are shown in Table 3. There was no significant difference in the expressivity as measured by the tumor laterality whether they received the mutated RB gene from father or from mother, indicating no differential expression associated with the parental origin.

Alternatively, male-derived mutation may be ascribed to the difference in the number of cell divisions during the production of germ cells between two sexes. If such is the case, we would expect the paternal age effect on the birth of the patients. However, it was not so, since we could not find any increase in the paternal age at all in any of the three groups of retinoblastoma patients; patients due to paternally derived *de novo* chromosome mutations, those with sporadic bilateral tumors, and those with sporadic unilateral tumors (Table 4). Therefore, the mutations are likely to stem from the post-meiotic stage of the male genome even including early stages of embryogenesis.

Table 3. Parental Transmission of RB Mutation and Expressivity in the Carrier Offsprings

Expressivity in carrier parents	Expressivity in carrier offsprings			
	Paternal transmission		Maternal transmission	
	Unilateral	Bilateral (%)	Unilateral	Bilateral (%)
Non-manifest	41	43 (51.2)	20	25 (55.6)
Unilateral	23	66 (74.2)	15	57 (79.2)
Bilateral	11	61 (84.7)	2	26 (92.2)
Total	75	170 (69.4)	37	108 (74.5)

Table 4. Paternal Age at Birth of Sporadic Patients with Retinoblastoma

	Hereditary		Non-hereditary
	13q-	Others	
No. of patients	10	225	408
Mean age (yrs)	30.2	30.2	30.2
Expected	30.3	30.1	30.1

13q- includes 4 cases of translocations.

The preferential paternal origin has been also known in other *de novo* chromosome structural rearrangements²⁶⁾. The mechanism of the preferential involvement of paternal genome in the chromosomal as well as subvisible mutations is not clear. The situation in male germ cells is complex. The late spermatid and spermatozoa are in the stage where DNA undergoes an extensive condensation by replacing chromosomal histone with protamine, deficient in DNA repair, and chemical modifications of DNA readily results in DNA strand breakage or accumulation of the stresses in the chromatin²⁷⁾. The male-directed mutagenesis could be intrinsic to such chemical and physiological property of sperm chromosomes. Indeed, human sperm chromosomes have been demonstrated to be highly vulnerable in terms of the spontaneous and radiation induced chromosome aberrations^{28,29)}.

Mutational Susceptibility or Mutational Mosaicism by Delayed Mutation

The preferential involvement of paternally derived chromosomes in the initial somatic mutation is even more puzzling. There is now an increasing evidence indicating that the parental-sex specific modification of genome persists after fertilization and expressed differentially during embryonic development³⁰⁾. Such male-specific modification could be related with the mutational susceptibility in the somatic cells.

However, it should be also noted that the mutagen treatment of post-spermatogonial germ-cell stages results in a high frequency of mutational mosaicism. Damage induced in sperm DNA may not necessarily be fixed as true mutation at the time of fertilization but often stays as premutation and be fixed as true mutation in the later stages of the embryonic development resulting in the mutational mosaics. The importance of such mutational mosaicism in sporadic mutation was first emphasized by Muller (1920)³¹⁾, and later elegantly demonstrated by Auerbach (1964)³²⁾. Auerbach³²⁾ postulated "premutations" which are labile and either disappear or could be fixed as true mutation during later stages of development even after generations. Recently, Favor *et al.* (1990)³³⁾ reported that in the F₁ of male mice treated with ethylnitrosourea at the post-spermatogonial stages, majority of mutations occurred as mosaics.

Possible mutational mosaicism has been discussed to explain the contiguous gene syndrome of man, which show wide spectrum of variability in phenotype^{34,35)}. The diseases

in this category are usually sporadic but occasionally familial. Mutational mosaicism has been also implicated to the development of sporadic unilateral retinoblastoma by Carlson and Desnick (1979)³⁶. Hermann (1977)³⁷ hypothesized the Auerbach-type delayed mutation model to explain the atypical inheritance of retinoblastoma.

Mutational mosaicism by delayed mutation of this sort is very attractive in explaining the strong bias towards paternal chromosomes in the germinal and initial somatic mutations. A possible mechanism is shown in Fig. 3. DNA damage or other DNA stresses which are carried over by sperm are the subjects to be repaired in the fertilized egg, where they are either repaired or fixed as true mutation or premutation. The true mutation will give rise to the hereditary retinoblastoma and can be transmitted to the next generation. During later stages of embryonic development, the persistent damage or premutation will be also fixed as true mutation or erased and eventually give rise to the mosaics of the cells having premutation, mutation and normal alleles. Because of the mosaicism, the premutation or mutation will be transmitted to the next generation only occasionally. The transmission of premutation may explain anomalies in familial retinoblastoma inheritance such as (1) atypical, collateral type pedigrees^{37,38}, (2) the appearance of retinoblastoma and osteosarcoma in the same family³⁹, and (3) increased risk of non-ocular cancers in the parents of sporadic unilateral retinoblastoma patients^{40,41}.

It is thus hypothesized that a majority, if not all, of the so-called non-hereditary sporadic unilateral retinoblastoma, possibly also Wilms tumor, rhabdomyosarcoma and osteosarcoma, also arises as a consequence of mutation originating from the post-meiotic male germ cells. The number of the cells in the body is far above the denominator of the mutation rate. The mutational mosaicism for the genes of usual monogenic syndrome will lead to the localized manifestation of the abnormalities or malfunctions and result in the

SEX-SPECIFIC MODIFICATION OF GENE AND FIXATION OF MUTATION

Fig. 3. Germ-cell origin and mutational mosaicism in the development of bilateral (B) and unilateral (A) retinoblastoma. Damage carried over by sperm chromosomes is fixed either into true mutation to give rise to hereditary retinoblastoma (B) or into premutation (or remained as persistent damage) which is further fixed into true mutation or erased to give rise to variegated mutational mosaicism which is manifested as non-hereditary unilateral retinoblastoma.

variegation of the disease or often escape the clinical manifestation. However, for the tumor-related genes, such mutation will directly link to the development of cancers. Therefore, the mutation rate basing on the conventional mutation approaches will be underestimated when it is applied to the estimate of cancer risk from the parental exposure to radiation and chemicals.

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