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Loss of Retinoblastoma Gene and Amplification of N-myc Gene in Retinoblastoma

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We have analyzed paired samples of genomic DNA from peripheral leukocyte and primary tumor tissue from nine patients with retinoblastoma (RB) and from two RB cell lines, WERI-Rb-1 and Y79, to detect the molecular alterations of the retinoblastoma susceptibility gene (RB-1) and N-myc gene. In Southern analysis, RB-1 deletions in tumor tissues were detected in five patients (56%), one of these revealed a total loss of RB-1. N-myc amplification was found only in one (11.1%) out of nine patients. We also observed a total loss of RB-1 in WERI-Rb-1, and a more than 100-fold amplification of N-myc in Y79. The analysis of the relationship between molecular events and clinical characteristics such as age, sex, tumor laterality did not reveal any specific correlation. These results suggest that genetic backgrounds of RB in Korean patients are quite similar to those of reported cases elsewhere. The high sensitivity of our methold in detecting the RB-1 loss indicates that this method can be a useful tool for initially screening a large number of tumors.

Key Words: *retinoblastoma, RB-1 gene, deletion, N-myc gene, amplification.*

INTRODUCTION

Retinoblastoma is the most common primary ocular neoplasm occurring in early childhood and more than 90% of patients are diagnosed before the age of five. Clinically, RB has two forms, hereditary and non-hereditray. The molecular pathogenesis of both forms has been explained by an elegant hypothesis by Knudson (1971) i.e., that two independent mutations leading to the inactivation of the tumor suppressor gene might play a major role in the malignant transformation of a retinal cell. This hypothesis was strongly supported by the subsequent cytogenetic analyses which showed a microscopically visible deletion of chromosome 13q 14 in RB tumor tissues (Yunis et al., 1978; Cavenee et al., 1983; Godbout et al., 1983). The ultimate isolation of a responsible gene, designated RB-1, allowed remarkable progress in the understanding of the structural and functional nature of RB-1 (Friend et al., 1986; Gallie et al., 1991) and of its alterations in human carcinogenesis.

The amplification of N-myc gene in RB was initially suggested as a critical step suggesting a possible interaction between production of RB-1 and N-myc (Lee et al., 1984). Alternatively, Squire et al. (1986) raised the argument of the possibility of N-myc as a functional target gene for RB protein. This question is still open to further investigation.

Despite numerous reports on the genetic backgrounds of RB, there has been no report analyzing the molecular pathogenesis of Korean RB. The assumption that similar alterations of RB-1 and N-myc found in other countries might be also involved in Korean RB is persuasive. Anyhow, we decided to confirm this because we could not rule out the possibility that a geographic difference may exist. We have analyzed primary tumor samples taken from nine patients with special interest to RB-1 and N-myc. We report here our results

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that the molecular backgrounds of Korean RB are quite similar to other cases reported elsewhere.

MATERIALS AND METHODS

Purification of genomic DNA:

Genomic DNA was isolated from peripheral leukocyte and RB tumor tissue of nine patients who were admitted to the Korea Cancer Center Hospital and from RB cell lines (WERI-Rb-1 and Y79; kindly provided by Dr.M. Inomata) by standard method of phenol and chloroform extraction followed by ethanol precipitation (Maniatis et al., 1989).

Preparation of probes:

The DNA probes used for the analyses of RB-1 and N-myc were H3-8 and pNB-1, respectively, purchased from the American Tissue Culture Collection. DNA fragments carrying RB-1 and N-myc were isolated by glass beads method using a Geneclean II kit (Bio 101 Inc. USA). Briefly, the DNA band visualized in agarose gel was excised and mixed with three volumes of NaI solution. After incubation at 50°C for five minutes, glassmilk suspension was added. Further incubation on ice was performed for five minutes and the pellet was recovered by a brief centrifugation. After washing twice with washing solution, the DNA insert was recovered by incubation at 50°C in TE solution for three minutes. The relevant size of insert was confirmed by agarose gel electrophoresis and the insert was used for the labelling.

Southern blot analysis:

About 15 μg of genomic DNA was digested with EcoRI restriction enzyme (BRL, USA) over 12 hours in a 37°C water bath. Digested DNA was separated by gel electrophoresis in 0.8% agarose and was transferred to a nylon membrane (Sigma, USA) using the standard Southern blot method (Maniatis et al., 1989). The blot was fixed by baking in an 80°C oven for two hours. The DNA probe was labelled with α-^{32} PdCTP by random primer method using the multiprime DNA labelling system (Amersham, USA). Hybridization was performed for 20 hours at 42°C in hybridization solution (6xSSC, 0.5% SDS, 100 μg/ml salmon sperm DNA, 50% formamide). The filter was washed at high stringency (0.1xSSC, 0.1% SDS, at 68°C) and was exposed to x-ray film (Agfa, Belgium) for an appropriate time at -70°C.

RESULTS

The Southern analysis for RB-1 with DNA from peripheral leukocyte showed three bands befitting 10.8 kb, 8.5 kb, and 5.7 kb or two lower bands in some cases suggesting a genetic polymorphism. In Figure 1, the results of four representative cases among nine cases analyzed and of two cell lines were presented. In the first case from the left side, two bands at 10.8 kb and 8.5 kb in control lane (N) were deleted in the tumor lane (T) representing a loss of heterozygosity. Similarly, in the second case, the 8.5 kb band which was seen in the control lane was deleted in the tumor lane. The faint band seen in the tumor lane seems to be resulted from contaminated normal tissue from the margin of the tumor. In contrast, no deletion was found in the third case. In the fourth case, total deletion of RB-1 was found. The total absence of RB-1 in the

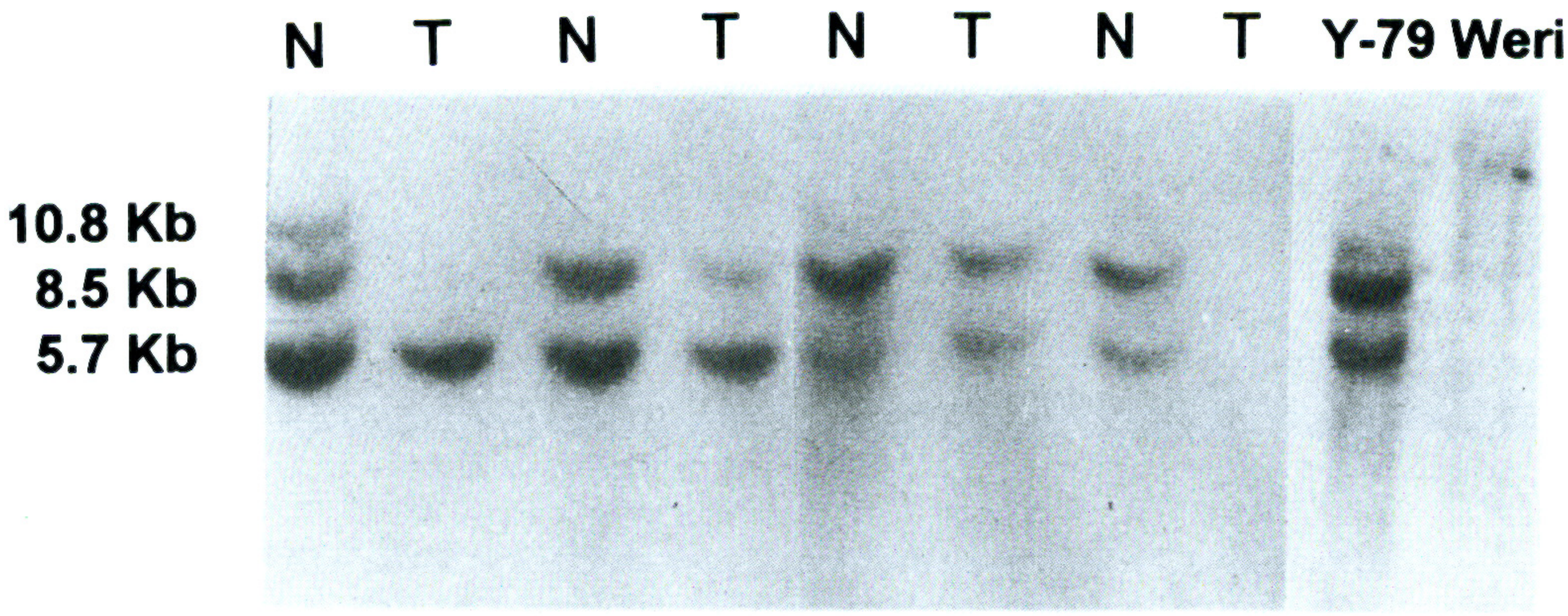

Fig. 1. RB-1 deletion in RB patients and two RB cell lines (Y79 and Weri-Rb-1). Of nine cases analyzed, four representative cases are presented here. N: control, T: RB tissue.

tumor lane of the fourth case was confirmed by repeated examination with the loading of a higher amount of DNA (data not shown). Total deletion of RB-1 in WERI-Rb-1 cell line, but not in Y70 was also observed. Of the nine patients we evaluated, five cases (56%) have shown RB-1 deletion in their tumor tissue compared to their controls (Table 1).

In Southern analysis for N-myc (Figure 2), two representative cases were presented. Only one of nine, the second case in Figure 2, was identified to have an approximately 20-fold amplification of N-myc gene. More than a 100-fold amplification was also found in the Y79 cell line as previously reported.

The clinical characteristics and results of molecular analyses of nine patients have been summarized (Table 1). The ages of the patients ranged from 2 months to 6 years. Six of them were males and three were females. One patient had tumors on both eyes. In four cases, RB developed in the right eye and in the other four, in the left side. No significant finding in terms of the correlation between clinical profile and molecular events was found.

Table 1. Clinical characteristics and genetic alterations in nine RB patients.

Patients	Age (year)	Sex	Laterality of tumor	LOH of RB-1	N-myc amplification
1	0.5	F	Lt	+	−
2	3.8	M	Rt	+	−
3	4.4	M	Rt	+	−
4	6.3	F	Lt	−	−
5	3.1	M	Lt	+	+
6	1.1	M	Lt	−	−
7	1.6	F	Rt	+ *	−
8	0.2	M	Rt	−	−
9	2.2	M	Both	−	−

LOH: loss of heterozygosity, *: total loss of RB-1.

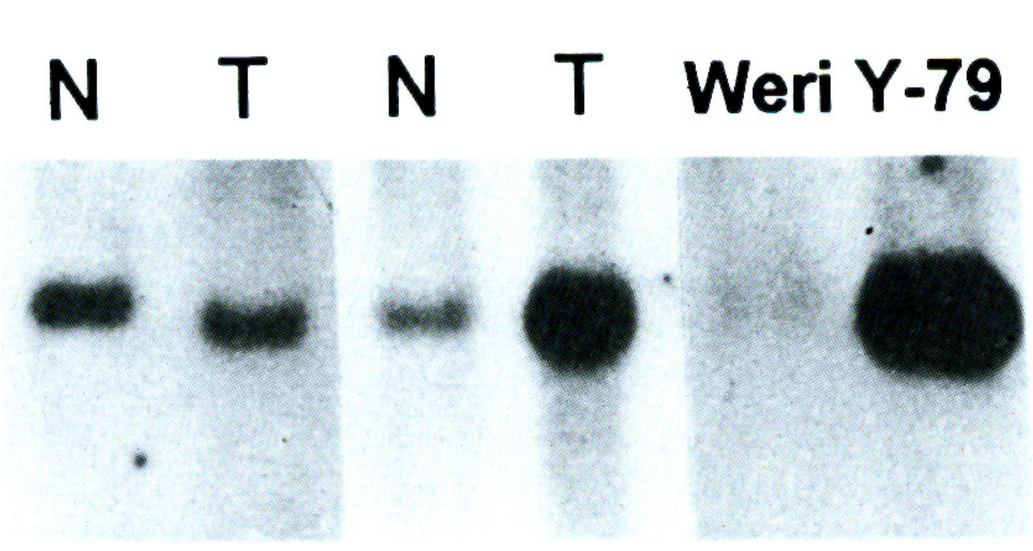

Fig. 2. N-myc amplification in RB patients and two RB cell lines (Weri-Rb-1 and Y79). Of nine cases analyzed, two representative cases are presented here.

DISCUSSION

Retinoblastoma susceptibility gene is the prototype for the tumor suppressor gene that acts normally to constrain a cellular proliferation (Sager, 1989). The total size of RB-1 in human genome spans 200 kilobases on chromosome 13q 14, and it's expression results a 4.7-kb mRNA transcript and a 110-kd nuclear phosphoprotein ($p110^{RB}$) with DNA binding activity (Yandell et al., 1989). The mechanism of RB-1 function in normal and malignant cells has been suggested by the ability of $p110^{RB}$ to bind with oncoprotein of DNA tumor virus such as adenovirus, SV40, human papilloma virus (Horowitz et al., 1990) and by the cyclic phosphorylation in a cell cycle (Cooper et al., 1989). The role of $p110^{RB}$ in a normal cellular environment seems to be a kind of "checking point" for abnormal proliferation. The breakdown of normal $p110^{RB}$ function by various genetic alterations such as chromosomal deletions (Friend et al., 1986) or point mutations (Dunn et al., 1989) results in an unlimited cellular proliferation or cell division leading to the malignant transformation of normal cells.

The genetic rearrangement of RB-1 in RB is believed to be the fundamental step in the malignant process of the retinal cell. Though the percent of RB-1 alteration in early series of experiments ranged only from 8-30% (Friend et al., 1987; Fung et al., 1987), recent data using a polymerase chain reaction or RNase protection assay increased the incidence of RB-1 mutation in RB tissues up to 70% (Dunn et al., 1989). The universal implications of RB-1 inacitvation in various tumors and tumor cell lines have also been demonstrated by an immunoblot analysis (Horowitz et al., 1990). Fifty six percent of RB-1 deletion in our experiment is definitely higher than those of pervious reports using a Southern analysis. We believe that the high incidence of RB-1 in our cases results from the difference in using the probe and restriction enzyme rather than from a real difference in terms of RB-1 alteration. The incidence of total deletion, one of nine, is comparable to Dryja's report which demonstrated homozygous deletion in two out of 37 RB tumors (Dryja et al., 1986). Because of the complexity of a RNase protection assay or an immunoblot analysis, Southern blot analysis having the acceptable sensitivity could be regarded as a good tool for initial screening to detect RB-1 alteration in a large number of cancers. It is well known that variable genetic alterations ultimately leading to the inactivation of wild type RB-1 are involved not only in RB but also in other human malignancies such as osteosarcoma, small-cell lung cancer, and

breast cancer (Toguchida et al., 1988; Weichselbaum et al., 1988; Harbour et al., 1988; Mori et al., 1990; Lee et al., 1988). The fact that P^{53} is associated in most common cancers (Sagér, 1989) also supports the view that tumor suppressor genes are involved in the malignant process of most cancers. It is highly expected *that extensive investigations may reveal the protean* involvement of RB-1 or it's gene product in most human cancers.

N-myc is a proto-oncogene normally expressed in the early stage of development in multiple tissues, including a fetal retina. Genomic amplification of N-myc has been restricted primarily to neuroectodermal tumors (Schwab et al., 1983; Nau et al., 1985), and many correlate with poor clinical prognosis (Seeger et al., 1985). An early study has proposed N-myc as a possible target for regulation by RB protein (Lee et al., 1984). They observed the N-myc amplification and increased expression in two and seven of ten, respectively. In contrast, another study (Squire et al., 1986) demonstrated that N-myc was expressed in fetal but not in adult brain and retina and in near-diploid RB samples at levels similar to those observed in normal fetal retina. Only two of 10 RB tumors exhibited increased levels of expression with genomic amplification of N-myc. Therefore, they concluded that N-myc expression in RB probably reflected the origin of tumor from an embryonal tissue, and was not directly associated with RB-1. In our study, N-myc amplification was only found in one of nine cases. These data rather support Squire's opinion that N-myc may not be directly related to the RB-1. However, we need further analyses of the transcript dealing with a large number of tumors to conclude this argument.

Our results which analyzed RB-1 deletion and N-myc amplification in Korean patients are quite similar to those reported elsewhere (Cavenee et al., 1983; Friend et al., 1986). This suggests that there may be no geographic difference in the molecular pathogenesis of RB. Although no clear association between clinical features and genetic alteration was found, the number of patients in our study was too small to make any conclusion regarding the clinical relationship. We hope that a large-scale and long-term follow-up study may reveal any clinical significance of genetic alteration. Furthermore, the necessity of four to five steps for malignant transformation in various human malignancies validates further efforts to find a novel oncogene or a tumor suppressor gene implicated in RB.

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