Abnormal Gene Expression Profiles in Unaffected Parents of Patients with Hereditary-Type Retinoblastoma

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Abstract

The hereditary form of retinoblastoma (Rb) is associated with a germ line mutation in one Rb allele and is characterized by the occurrence of multiple, bilateral Rb tumors and a predisposition to the development of second cancers. In an earlier study, we observed an unexpected hypersensitivity to ionizing radiation in skin fibroblasts derived from unaffected parents of children with hereditary Rb. In at least four of these five families, there was no family history of Rb, indicating a new germ line mutation. We hypothesize that the increased parental cell sensitivity to radiation may reflect the presence of an as yet unrecognized genetic abnormality occurring in one or both parents of children with Rb. In the present study, we use DNA microarray technology to determine whether differences in gene expression profiles occurred in the unaffected parents of patients with hereditary Rb relative to normal individuals. Microarray analyses were validated by quantitative reverse transcription-PCR measurements. A distinct difference was observed in the patterns of gene expression between unaffected Rb parents and normal controls. By use of the prediction analysis for microarrays and principal component analysis methodologies, significant differences between the two groups were identified when as few as nine genes were analyzed. Further study of this phenomenon may offer a new insight into the genetic mechanisms of Rb and perhaps more broadly in cancer biology. (Cancer Res 2006; 66(7): 3428-33)

Introduction

The hereditary form of retinoblastoma (Rb) is associated with a germ line mutation in one of the Rb genes and is characterized by the occurrence of multiple, bilateral Rb tumors and a predisposition to the development of second cancers. These second tumors often occur within the radiation field in patients treated by radiation therapy (1). Interestingly, however, the family history is often negative for Rb, suggesting that a new mutation has occurred in the parental germ line. In a high percentage of cases, such new mutations arise in the paternally derived allele (2, 3). However, parental mosaicism of a mutation arising during early embryonic development of the zygote may also occur (4). Among Rb patients with high-penetration, germ line mutations, no known genetic abnormalities have as yet been identified in the somatic cells of their parents, although a statistically significant increase in the incidence of cancer has been reported (5–7).

In an earlier study, we observed an unexpected hypersensitivity to ionizing radiation in skin fibroblasts derived from unaffected parents of children with hereditary Rb (8). In at least four of these five families, there was no family history of Rb, indicating a new germ line mutation. Enhanced radiosensitivity was manifested by both an enhanced radiation-induced G1 arrest and an increased sensitivity to cell killing as measured by a clonogenic survival assay. We hypothesize that the increased parental cell sensitivity to radiation may reflect the presence of an as yet unrecognized genetic abnormality occurring in one or both parents of children with Rb. Interestingly, the radiosensitivity of two of the five probands studied fell within the reference range, consistent with earlier reports showing variability in the response of cells from patients with hereditary Rb to ionizing radiation (9, 10).

In the present investigation, we used microarray technology to determine whether differences in gene expression profiles occurred in skin fibroblast cell strains isolated from unaffected parents of patients with hereditary Rb compared with cells from normal individuals. Although initial experiments were carried out using pooled RNA from all family members, including probands, the more detailed analysis focused on cells from individual parents analyzed separately. Extensive cell culture collections from Rb families are not generally available; thus, the availability of cells from the parents of Rb patients was an unusual feature that allowed this study to be carried out. A distinct difference was observed in the patterns of gene expression between the fibroblasts from unaffected Rb parents and normal controls. By use of the prediction analysis for microarrays (PAM) and principal component analysis (PCA) methodology (11, 12), significant differences in expression profiles between the parental fibroblasts and normal controls were identified when as few as nine genes were analyzed.

Materials and Methods

Cell culture. Clinical information and radiosensitivities concerning the skin fibroblast cell strains from the parents and probands from five Rb families, along with the range of radiosensitivities from numerous low passage normal human fibroblasts was reported previously (8). Briefly summarizing the radiosensitivities as measured by the dose required to reduce cell survival to 10% (D10) from this report, the cells derived from
parents of Rb probands yielded $D_{M}$ estimates ranging from about 1.0 to 2.3 Gy, whereas most of the cell strains derived from apparently normal individuals ranged from about 2.6 to 3.1 Gy. Interestingly, $D_{M}$ values in the reference range were observed for cells from probands from families II and IV (2.7 and 2.8 Gy, respectively), whereas $D_{M}$ values for cells from the probands for families I, III, and V (2.1, 2.0, and 1.3 Gy, respectively) were in the hypersensitive range seen for unaffected parents (8). The cell strains from Rb families were all established at the Harvard School of Public Health and have been designated according to the five families of origin as MF-4E, MF-2M, and MF-3R from family I; MF-6F and MF-7R from family II; MF-11F, MF-12M, and MF-10R from family III; MF-15F, MF-13M, and MF-14R from family IV; MF-18F, MF-16M, and MF-17R from family V, where in each family designation the final code letter F, M, or R, respectively, refer to the father, mother, or proband (8). The low passage normal fibroblast culture used in this study were from the National Institute of General Medical Sciences Human Cell Repository at the Coriell Institute for Medical Research (CIMR) in Camden, NJ (8). For the present study, 12 low-passage fibroblast cell strains from apparently normal individuals also from the CIMR were used as controls. These strains are designated GM00041, GM00969, GM02673, GM03440, GM04501, GM04503, GM05576, GM08333, GM08447, GM08429, and AG01521 and were age and sex matched with the CIMR were used as controls. These strains are designated GM00041, GM00969, GM02673, GM03440, GM04501, GM04503, GM05576, GM08333, GM08447, GM08429, and AG01521 and were age and sex matched with those to from the Rb family members and studied at generally similar passage levels. Exponentially growing cells were maintained in Eagle's MEM (Life Technologies, Grand Island, NY) supplemented with 15% heat-inactivated fetal bovine serum, penicillin (50 units/mL), and streptomycin (Life Technologies, Grand Island, NY) supplemented with 15% heat-inactivated fetal bovine serum, penicillin (50 units/mL), and streptomycin (100 μg/mL). Cell cultures grown to 80% confluence in P-100 plastic dishes were subcultured into larger P-150 plastic dishes. When these cultures reached ~70% confluence, the medium was renewed, and RNA was isolated 24 hours later when the cells had resumed exponential growth.

**RNA extraction and purification.** Cells were washed twice with ice-cold PBS; 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA) was added to cell pellets, which were vortexed and incubated for 5 minutes at 4°C. The mixtures were transferred into 1.5-ml centrifuge tubes and 200 μL of chloroform per mL of Trizol was added to partition the phases. The mixtures were iced for 5 minutes and then centrifuged (10,000 rpm, 4°C) for 10 minutes. The aqueous layers were then transferred into new 1.5-ml centrifuge tubes, and a 0.5 volume of 2-propanol was added. Samples were pelleted by centrifugation (10,000 rpm, 4°C) for 10 minutes, then in 0.2% SDS), the entire sample (40 μL) was loaded onto the slides. Hybridization was carried out in a humid chamber overnight in a 42°C water bath.

**Washing, scanning, and image analysis.** Following hybridization, the slides were washed in first wash buffer (1× SSC, 1% bovine serum albumin) at 42°C for at least 1 hour. Slides were washed in deionized water followed by 2-propanol. Cy5- and Cy3-labeled samples were mixed with 1 μL each of human Cot1-DNA (10 μg/μL; Invitrogen), poly(A) (8-10 μg/μL; Amersham Pharmacia Biotech, Piscataway, NJ), and yeast tRNA (4 μg/μL; Ambion, Austin, TX) for hybridization. The mixed samples were denatured at 100°C for 1 minute, and after the addition of 20 μL of 2× hybridization buffer (50% formamide, 10× SSC, 0.2% SDS), the entire sample (40 μL) was loaded onto the slides. Hybridization was carried out in a humid chamber overnight in a 42°C water bath.

**Real-time reverse transcription-PCR assay.** After an initial expression analysis, seven clones were selected for validation of cDNA microarray data by use of a reverse transcription-PCR (RT-PCR) assay. In addition, one clone (GADPH) was selected as a control gene. Quantitative RT-PCR was done by taking 1 mg of total RNA, which was reverse transcribed with Superscript II enzyme (Invitrogen), Real-time PCR was done with the ABI Prism 7900HT II sequence detection system with reagents purchased from PE Applied Biosystems (Foster City, CA). Primer sequences were designed using Primer Express software (PE Applied Biosystems). Amplicons were 50 to 150 bp long.

**Data analysis.** Data were corrected by median centering. Clustering was done by use of Eisen's Cluster and TreeView. For unsupervised clustering, genes were chosen based on the PAM result and were from the entire sample set. PCA was done to visualize the similarity of the log-transformed expression ratios of genes. PCA reduces the dimensionality of the data (expression profile of 19,000 genes) into three dimensions by summarizing the most important (i.e., defining) parts while simultaneously filtering out noise.

Supervised classification prediction analyses were done by applying PAM software hosted on National Cancer Institute's mADb server (http://nciarray.nci.nih.gov/). The method of the nearest shrunk centroids identifies a subgroup of genes that best characterizes a predefined class (12). The prediction error was calculated by means of 10-fold cross-validation. The model is fitted on 90% of the samples, and the class of the remaining 10% is then predicted. This process is repeated 10 times, with each part playing the role of the test samples and the error of all 10 parts added together to compute the overall error. The error within the validation set reflects the number of samples wrongly predicted to be in this set.
Results

As previously reported (8), all of the cell strains derived from the unaffected parents in the Rb families, as well as strains from three of the five probands (families 1, 3, and 5), showed a moderate degree of hypersensitivity to γ-radiation. All of the probands were classified as having hereditary-type Rb, based on the occurrence of multiple bilateral tumors. With the exception of Family 3, family histories were negative for Rb or other known cancer-susceptibility disorders. Thus, at least four of the cases represented new germ line mutations; the specific mutations were previously described (8). The father in family 3 had one eye enucleated at the age of 2 years for a single, unilateral Rb tumor.

In a pilot experiment, RNA samples extracted from all 14 cell strains derived from the Rb family members were pooled and compared directly with pooled RNA from 11 cell strains from apparently normal individuals by microarray analysis. A subset from the microarray analysis was then further analyzed using quantitative RT-PCR. Fifteen genes were identified through cDNA arrays, and 22 through oligo arrays that showed >2-fold increased or 2-fold decreased expression in the pooled RNA samples from the Rb families compared with the normal controls (data not shown). Among these genes, six showed significant changes in expression in both cDNA and oligo arrays. These six genes (i.e., IGFBP5, IGFBP7, ALDH1A3, JAG1, COL4A2, and COLA5A3) were selected for further analysis by quantitative real-time RT-PCR. One additional clone (GAPDH) was employed as an unrelated housekeeping gene. The results from the cDNA microarray and quantitative RT-PCR analyses are shown in Fig. 1. As can be seen in Fig. 1, there was excellent agreement between differences in expression for these genes as measured by the cDNA microarray and RT-PCR analyses. Similar agreement was shown for the oligo microarrays (data not shown). The excellent agreement between both cDNA and oligo microarray data and RT-PCR analysis thus validates the techniques for measuring expression levels in the present study. It is noteworthy that the expression level of the GAPDH housekeeping gene was not sufficiently higher than that necessary to reach the criterion of a 2-fold change considered to be significantly different from normal background fluctuations.

![Figure 1. Validation of cDNA microarray data by RT-PCR. Expression ratios between the pooled RB samples relative to the pooled normal samples were compared using cDNA microarrays (filled columns) or quantitative RT-PCR (open columns) for each of seven selected genes. A ratio \( >1 \) indicates upregulation in the pooled RB samples, whereas a ratio below \( -1 \) indicates down-regulation. GAPDH was used as an unrelated housekeeping gene whose relative expression level, as expected, was not significantly different from normal variations in expression levels, in keeping with the 2-fold change criterion usually applied to judge significant changes.](image)

Because the focus of this study is on possible differences in gene expression for cells from a normal control population relative to the parents of Rb patients and not the patients themselves, a comparison of gene expression in cells from individuals rather than the pooled averages was considered likely to be more informative. Thus, in the following experiments, RNA samples from the nine cell strains derived from the parents in the Rb families and the 12 apparently normal cell strains were analyzed individually by use of oligo microarrays. To compare gene expression profiles between the RB and normal RNA samples based on global gene expression data, the PCA method was employed. PCA reduces the dimensionality of the 19,000 genes in this study to only very few dimensions, by summarizing the most defining parts while simultaneously filtering out noise components. With the analysis of all 19,000 genes, the difference between the RB parental and normal samples was immediately evident; the RB and normal samples formed two distinct groups on the right and left side of the first PCA dimension (data not shown). This clear separation of samples, which implied the existence of an inherent difference between them in their gene expression profiles, led to our next step, in which we identified a much smaller set of genes to be used for future grouping.

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<th>Shrinkage Delta</th>
<th>No. Genes</th>
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We applied the method of PAM, in which samples were divided into training and testing sets instead of training sets alone. This was designed to avoid bias prediction and to estimate error rate. Among the 19,000 genes examined, nine sets of genes were identified, all with the lowest misclassification error of 0.048. The number of genes in those sets ranged from 9 to 289 (Table 1). Genes in the smaller sets are wholly included in the larger sets. These sets...
were then examined to determine the smallest number of genes that can separate clearly the two groups of samples.

PCA analysis of the above nine sets of genes showed that the one with 42 genes most effectively separated the Rb cell strains from the normal strains. These results are shown in Fig. 2A. As can be seen, the nine samples from the Rb parents (green spheres) clustered closely together and were distinctly different from those of the 12 normal controls (red spheres). The set with only nine genes also showed clear separation between the RB parents and normal controls, with the exception of a single control strain (GM00969), which seems closer to the cluster of RB samples than normal controls (Fig. 2B).

The 42-gene set was further analyzed by use of the hierarchical cluster mapping method (Fig. 2C). Samples can be grouped into two distinct clusters, one RB parent cluster and one normal cluster. Each cluster displayed a pattern that seems to be different from the other, and these patterns were consistent among most of the samples within each group. Interestingly, normal control strain GM00969, while being clustered into the normal group, displayed some patterns that were more similar to the RB samples than to the remainder of the control group. The examination of the array cluster indicated that this individual had the least linkage to the rest of the control group and was positioned on the border of the cluster of RB samples. The same individual seemed to be outside of the core cluster by PCA analysis (Fig. 2A and B).

Discussion

Microarray experiments result in a large number of data points that require extensive data mining and analysis to retrieve useful information for resolving biological questions. Therefore, the development of robust analytic tools for microarray data is very important. PCA has been widely employed as an analytic tool for this purpose. For example, Huang et al. (15) used PCA to...
distinguish different hepatotoxic agents and toxicity end points based upon gene expression data. PCA was also used to identify a set of genes that may play a role in tumor development (16). The analysis of global expression patterns by this approach in the present study yielded a clear indication that skin fibroblasts isolated from parents of patients with Rb have an inherently different gene expression profile than cell strains derived from apparently normal individuals. The subsequent identification of 42 genes or 9 genes provides a simpler method to differentiate the two groups and makes such separation more obvious. Identification of these small subsets of genes from global gene expression analysis may be used in the future as new biomarkers, including perhaps increased rates of germ line mutation at the Rb or other loci, possible changes in systems involved in genome stability, or increased sensitivity to radiation or other genotoxic agents.

Although the PCA analyses showed that there were distinct differences in gene expression profiles between the Rb parents and normal samples, one of the normal samples (GM00969) showed a genetic profile approaching that of the Rb parents. This raises the question as to whether other cell strains may show moderate hyper-radiosensitivity and may share certain genetic characteristics with the Rb parents. A corollary question is the extent to which the changes we have observed in specific gene expression may be related to the radiosensitivity phenotype rather than the Rb susceptibility phenotype. Interestingly, control strain GM00969 that displayed some gene expression patterns similar to the Rb samples was also moderately radiosensitive; however, two other control strains showed similar hypersensitivity, whereas their expression patterns clearly clustered with the Rb parents (data not shown). We are currently further investigating this question, including specifically an examination of gene expression profiles in the Rb probands, two of which were reported by Fitzek et al. to be normal (not hypersensitive) with respect to radiation sensitivity (8).

Among the 42 genes that were shown by PCA analysis to be the best set of genes for distinguishing normal control fibroblasts from RB family member–derived fibroblasts, NM23A, MCM5, HOXB2, HOXD10, HOXC10, polo-like kinase 1, and E2F1 are especially interesting because each of these seven genes has been shown to be involved in cancer development. All are associated with various aspects of cellular growth. These include cancer metastasis (NM23A; ref. 17), DNA replication (MCM5; refs. 18, 19), cellular differentiation (HOXB2, HOXD10, and HOXC10; refs. 20, 21), mitosis (polo-like kinase 1; ref. 22), and G1-S phase transition (E2F1; ref. 23). In all these cases, expression seemed to be decreased in cells from RB family members.

The exact biological significance of this gene expression pattern is not clear. The reduced expression of NM23A in the Rb parents seems plausible because it had been implicated as a tumor suppressor gene. The decreased expression of the other genes is, however, puzzling and counterintuitive as most of these genes have been associated with cellular proliferation and cancer development (18, 24–26). One might expect the expression of these genes to be increased in RB-associated fibroblasts, as the RB gene negatively regulates genes that are involved in cellular proliferation (e.g., E2F1), but in the present study, we are dealing with cells from unaffected parents who are not RB heterozygotes and are presumably normal for both copies of the RB gene. Another interesting observation is the absence of known DNA repair genes from the 42-gene list. Such genes might be among one group of logical candidates for parental hypersusceptibility to the development of new germ line RB mutations or hypersensitivity to radiation. This finding suggests that the moderate radiosensitivity observed in cells from the RB families is not directly related to significant alterations in the expression of a known DNA repair gene.

DNA microarray analysis done on clinical specimens provides information directly applicable to cancer diagnosis as well as disease outcome (27–32). Gene expression profiling of leukemia patients was employed to distinguished acute myeloid leukemia (AML) from acute lymphoblastic leukemia (ALL); in addition, it showed that a subset of leukemia with morphologically apparent ALL had a similar gene expression profile and therapy response to AML (28). Two classes of breast tumors with or without BRCA gene mutations can be classified by different gene expression profiles (29). In view of the different origins and diversity of genetic backgrounds for the normal and RB cell strains we used in the present study, it is worth noting that despite the different origins and genetic backgrounds for the cells used in the BRCA study, the different expression profiles were, nevertheless, clear (29). In another study, molecular profiles in esophageal squamous cell carcinoma were found to differ in familial cases compared with sporadic cases (32). A few studies have related tumor gene expression profiles to treatment responses and outcomes after surgery, radiation, or cytotoxic agents (33–36). A total of 92 genes selected as a discriminatory set predicted all responders after docetaxel treatment in 24 breast cancers (33). Genes related to tumor chemosensitivity were reported in patients with esophageal cancer (36). Fukuda et al. reported that genes related to apoptosis (BIRC2 and CASP6), inflammation (COX-2), DNA metabolism (CD73), cell growth (PLAU), cell adhesion (CDH1 and CDH3), transcription (MLL3), and cell cycle (CDK6) were expressed differently in radioresistant esophageal cancer cases compared with radiosensitive cases (35).

The present studies showing that there are significant differences in gene expression in somatic cells from unaffected parents of Rb patients compared with normal controls suggest that these parents may harbor some as yet unidentified genetic abnormality. Further study of this phenomenon may offer a new insight into the genetic mechanisms of Rb and perhaps more broadly in cancer biology.

Acknowledgments

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References

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