Title
Molecular genetic and clinical correlative studies of medulloblasstoma

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Molecular genetic and clinical correlative studies of medulloblastoma

by

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Abstract

Loss of heterozygosity for chromosome 17p sequences in conjunction with mutations in the human p53 gene have been demonstrated in a variety of human cancers. Because of the frequent cytogenetic finding of isochromosome 17q, we performed a molecular genetic analysis of chromosome 17p and p53 for medulloblastoma, a common childhood brain tumor. Using restriction fragment length polymorphism (RFLP) analysis, we demonstrated loss of heterozygosity for chromosome 17p sequences in 11 of 22 (50%) medulloblastoma patients. Of these eleven patients, all demonstrated loss of distal 17p13.3 sequences, and eight also lost more proximal 17p12 sequences with preservation of intervening markers. Deletion of 17p sequences correlated with poor clinical outcome. Using denaturing gradient gel electrophoresis (DGGE) and polymerase chain reaction (PCR) direct sequencing, the highly conserved regions of the human p53 gene (17p13.1) were examined for mutations. Two of the 22 patients were found to have point mutations. These results suggest that p53 mutations may contribute to the pathogenesis of medulloblastoma in some cases but that additional or alternative tumor suppressor genes on chromsome 17p may be involved.

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Introduction

Although primary brain tumors are the most common form of solid cancer in children (1), little is known about their etiology, and the prognosis for these patients remains poor. Our laboratory efforts have therefore been focused on understanding the molecular genetics of pediatric brain tumors. It is our hope that this knowledge will provide for novel treatment and better means of predicting clinical outcome so that we will eventually be able to improve the outlook for these children. This thesis concerns our current understanding of medulloblastoma, a common childhood brain tumor.

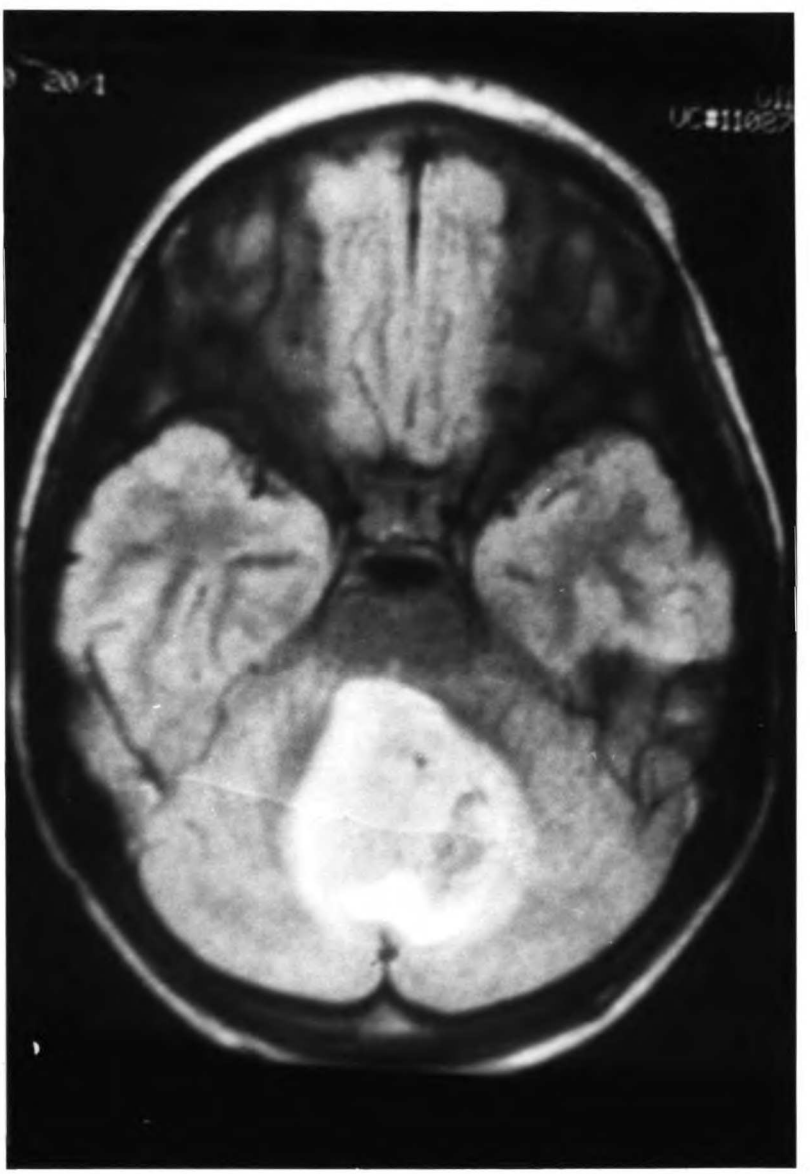
Medulloblastoma

Most pediatric brain tumors arise in the posterior fossa of the cranium (1). Medulloblastoma, the most common malignancy in this location, accounts for about 25% of all childhood brain tumors and has a peak incidence in the first decade of life (2). The tumors are soft, friable masses which are usually found in the midline of the cerebellum (Figure 1), often causing obstructive hydrocephalus. Typical presenting clinical signs and symptoms therefore include ataxia, headaches, nausea, vomiting, and papilledema. A unique feature of medulloblastoma is its propensity to spread throughout cerebrospinal fluid pathways. Metastatic lesions along the neuroaxis are therefore often found at presentation or in recurrent disease (2).

Current treatment for medulloblastoma includes as complete a surgical resection as possible followed by craniospinal irradiation. Those considered to be "poor risk" based on various clinical criteria (3) and those with recurrent disease also receive chemotherapy (2). Advances in both surgical technique allowing for complete resection of the tumor in most cases and the adjuvant use of radiotherapy and chemotherapy have clearly improved the survival for children with medulloblastoma (4). In 1930, Cushing reported a 32% operative mortality and only a single two-year survivor in his series of 61 medulloblastoma patients (5). By contrast, recent series using current treatment protocols have achieved overall five-year survival rates between 50% and 70% (6, 7, 8). Nonetheless, the clinical course and time to tumor progression for medulloblastoma patients is variable; some patients achieve stable disease following treatment, while others develop recurrent disease and die sooner. Given the long term adverse effects of adjuvant therapy on growth (9), cognitive

Figure 1: Cranial MRI of a medulloblastoma patient

The patient (Med-12) is a 5 year-old girl who presented with headaches, nausea, vomiting, and ataxia. The axial cut cranial MRI scan below demonstrates a large mass in the middle of the cerebellum. A gross total resection was performed and pathologic specimens confirmed the diagnosis of medulloblastoma. Genetic analysis in our lab demonstrated loss of heterozygosity for both proximal and distal 17p markers and the absence of p53 mutation. The patient died 14 months after diagnosis despite treatment with surgery, radiation therapy and chemotherapy.



development (10,11), and endocrine function (12), current treatment efforts aim not only to improve survival, but also to minimize toxicity in those with more favorable disease. Potential prognostic factors such as tumor histology (13, 14, 15), TNM staging (8, 16), extent of resection (6), and age (17) have been studied, but their ability to predict clinical outcome remains controversial.

Molecular genetics of cancer

It has been recognized for many years that genetic alterations play an important role in the development of cancer (18). Evidence included the recognition of familial cancer syndromes (neurofibromatosis, multiple endocrine neoplasia, familial adenomatous polyposis), the demonstration that known carcinogens are mutagens, and the high incidence of cancer in patients with defective DNA repair mechanisms (xeroderma pigmentosa). Oncogenesis is thought to be a multi-step process (18) in which progressive genetic damage results in the activation of growth-promoting genes and the inactivation of growth-suppressive genes.

In recent years, several of the genes involved in this multi-step process have been isolated and characterized. These genes can be classified as either dominantly acting oncogenes or recessively acting tumor suppressor genes (19). The oncogenes were discovered through studies of tumor viruses (20). Viral genes which caused transformation were isolated, and sequence analysis revealed that they were mutated versions of normal cellular homologs. Many of these "proto-oncogenes" were found to be cellular growth factors or their receptors which, when mutated, become overexpressed or devoid of their normal regulatory mechanisms (20).

Tumor suppressor genes are thought to function normally as negative regulators of cell growth, and thus inactivation of both copies of these genes in tumor cells is thought to result in neoplastic transformation (21). The existence of tumor suppressor genes was initially hypothesized by Knudson in 1971 (22). Based on epidemiological studies of familial and sporadic retinoblastoma, he proposed that two genetic "hits" could produce the tumor. In sporadic retinoblastoma, both mutations are acquired somatically; in familial retinoblastoma, one mutation is inherited and the other is somatically acquired.

Confirmation of this hypothesis later came through molecular genetic studies using restriction fragment length polymorphism (RFLP) analysis, which showed that specific chromosome 13 alleles present in control tissue were

deleted in the tumor specimen from the same individual (23, 24). These experiments thus suggested that after the first mutation is acquired, the remaining wild-type allele may be inactivated either through deletion or by homologous recombination with the chromosomal region containing the mutant allele. Because adjacent chromosomal regions may be involved in this reduction to homozygosity, anonymous DNA markers detecting heterozygous RFLPs may also show a parallel "loss of heterozygosity." Such RFLP analyses led to localization of the putative retinoblastoma (Rb) gene to chromosome 13q14 (23, 24). A gene was then identified which was found to contain point mutations in tumor specimens in which deletion of RFLP alleles could not be demonstrated (25, 26). This Rb gene has since been shown to encode a DNA binding protein (27).

Through RFLP analysis, tumor suppressor genes have been implicated as etiologic factors in a wide variety of human malignancies. These include other pediatric solid neoplasms such as Wilms' tumor (28, 29), adult solid neoplasms such as breast (30), colon (31, 32), and lung cancer (33, 34), and other adult brain tumors such as acoustic neuroma (35), meningioma (36, 37), and adult glioma (38). Since the discovery of Rb, other tumor suppressor genes have been cloned and characterized. These include the "deleted in colon cancer" or DCC gene on chromosome 18q21.3 (39), the neurofibromatosis gene (NF-1) on chromosome 17q11.2 (40), the Wilms' tumor gene (WT) on chromosome 11p13 (41), and the p53 gene on chromosome 17p13.1 (42). Of these known tumor suppressor genes, p53 has become the most widely studied, as point mutations in this gene have been demonstrated in diverse types of human cancer (43, 44, 45).

Specific Aims

Considerable progress has been made in our ability to understand the genetic events underlying the process of carcinogenesis for many types of human cancer. Nonetheless, little is known about the etiology of medulloblastoma on a genetic and molecular level. Cytogenetic studies of medulloblastoma have demonstrated that the most consistent karyotypic abnormality is the finding of isochromosome 17q, an abnormal chromosome that contains two long or "q" arms joined by a centromere with deletion of the two short or "p" arms (46). Because of this evidence for deletion of chromosome 17p material, we performed an RFLP analysis to determine if tumor suppressor genes might be involved in the oncogenesis of medulloblastoma. Furthermore, due to the frequent involvement

of p53 in other human malignancies and localization of this gene to chromosome 17p13.1, we performed a sequence analysis of the p53 gene in patients with medulloblastoma. Finally, we examined the relationship between the genetic data obtained and the clinical status of our patients to determine if this information may be useful in predicting clinical outcome.

Methods

Medulloblastoma patients

Twenty two patients with biopsy-proven medulloblastoma who underwent treatment at the University of California, San Francisco between 1988 and 1991 were included in this study. There were thirteen males and nine females with an age at diagnosis ranging from 9 months to 29 years (median 6 years). All patients underwent surgical resection followed by craniospinal irradiation plus a local boost to the posterior fossa. Patients initially classified as "poor risk" (Table 1) by a modification of the Chang criteria (3) and those who developed recurrences also received chemotherapy. After treatment, patients were then re-assessed clinically and with head and spine MRI at regular intervals (median follow-up time 19.5 months).

Freshly excised tumor specimens were obtained from each patient and flash-frozen in liquid nitrogen. As a control tissue, peripheral blood was also obtained from the same patient at the time of surgery. Protocols for collecting specimens were previously approved by the Committee on Human Research, University of California, San Francisco.

DNA Extraction

Total genomic DNA was extracted from the both the tumor and blood specimens from each patient. Twenty to thirty milliliters of whole blood was centrifuged at 1200 rpm for 15 minutes in order to isolate the buffy-coat containing peripheral blood leukocytes. DNA was then extracted from the leukocytes through lysis and treatment with SDS and proteinase K (47). Tumor specimens were manually disaggregated with a razor blade, and the DNA extracted using the same technique.

Unique Sequence Chromosome 17 probes

Table 2 shows the unique sequence DNA probes used in this study. All were previously known to detect restriction fragment length polymorphisms at various loci along the length of chromosome 17 and were either obtained directly from the investigator or through the American Type Culture Collection. The cloned sequences were transformed into competent host bacteria, and a large scale plasmid prep (48) was used to isolate several hundred micrograms of probe. The insert was then isolated from the plasmid vector by digestion with the

Table 1 - Criteria for determination of "poor risk" classification

Any one of the following:

1. Age less than 3 years
2. Total or near total surgical resection not achieved
3. Brain stem invasion
4. Spinal metastases
5. Positive CSF cytology

Table 2 - List of unique sequence DNA probes used in this study. Each is known to detect one or more RFLP at various chromosome 17p loci (56) using the restriction enzyme(s) listed. The probes were obtained through the American Type Culture Collection or directly from the investigator listed.

Probe	Location	Enzyme	Source
144-D6	17p13.3	Rsa I, Taq I, Msp I	M. Litt
JCZ16.2	17p13.3	Rsa I	R. White
YNH37.3	17p13.3	Msp I, Taq I	Y. Nakamura
YNZ22.1	17p13.3	Msp I, Bam HI	Y. Nakamura
EW502	17p13.2	Bgl II	E. Wright
HRP5.5	17p13.1	Hind III, Eco RI	R. Weinmann
MCT35.1	17p13.1	Msp I	Y. Nakamura
HP53b	17p13.1	Ban II, Bgl II	D. Givol
MYH2	17p12	Hind III, Msp I	L. Leinwand
HF12-2	17p12	Msp I	R. White
EW503	17p12	Msp I	E. Wright
EW409	17p12	Msp I	E. Wright
EW301	17p11.2	Bgl II	E. Wright
YNM67	17p11.2	Taq I, Rsa I	Y. Nakamura
UC10-41	17p11.2	Msp I, Pvu II	D. Barker

appropriate restriction enzyme(s), electrophoresis on a low-melting point agarose gel, followed by excision of the insert band with a razor blade. The excised band was rinsed several times with water, boiled for 10 minutes, and stored at -20 degrees C until use.

Restriction Fragment Length Polymorphism (RFLP) Analysis

Three micrograms of total genomic blood and tumor DNA from each patient was digested to completion with a restriction enzyme known to detect an RFLP with the probe of interest. The digested DNA samples were electrophoresed at 35 volts for 16 hours on 0.8% agarose gels, denatured, and transfered to a nylon membrane (Zetabind, AMF-CUNO) by Southern transfer (49). The resulting blots were then hybridized with probes that had been radiolabeled by the random priming technique (50). The blots were then washed to remove nonspecific binding and exposed to XAR-5 film (Kodak) for 3 to 14 days. Visual comparison was made between the signal seen for blood and tumor DNA from the same individual after it was verified that the size of the fragments present on the film were the same as previously described for the probe being examined. For each patient, the chromosome 17 probes were then assessed as being preserved (heterozygosity for the probe in both blood and tumor DNA), lost (heterozygosity in blood DNA, hemizygosity in tumor DNA), or noninformative (homozygosity in blood DNA).

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (51) was used to amplify various p53 gene fragments from the tumor and blood genomic DNA of each patient. Figure 2 shows the location of the primers with respect to the exon and intron structure of the human p53 gene. The primer sequences were as follows:

AOG 1+GC: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGGGATCCTTCCTCTTCCTGCAGTACTC

AOG 2: GCCGGAATTCAGTTGCAAACCAGACCTCAG

AOG 3+GC: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGGGATCCGTGTTGTCTCCTAGGTTGGCT

AOG 4: GCCGGAATTCCAAGTGGCTCCTGACCTGGA

AOG 5: GCCGGGATCCCCTATCCTGAGTAGTGGTAATC

AOG 6+GC: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGGAATTCCCCAAGACTTAGTACCTGAAG

AOG 7+GC: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGGGATCCTGGATCCTCTTGCAGCAGCC

AOG 8: GCCGGAATTCGGCAGGGTTCGTTACCTACTAAA

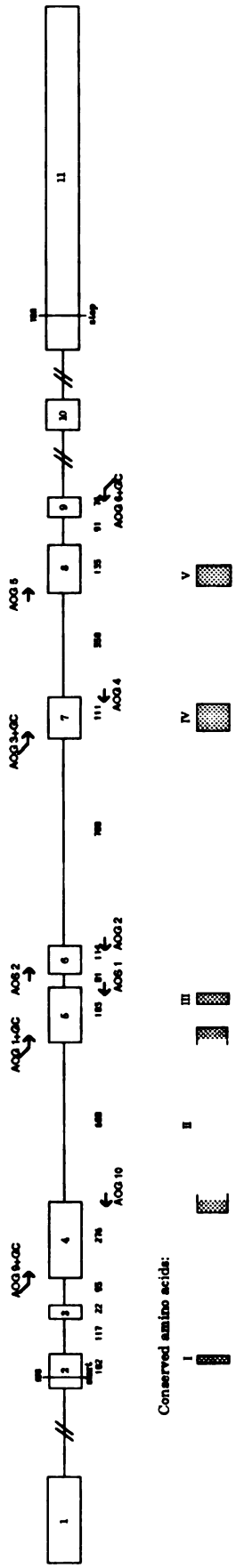


Figure 2: Human p53 gene and location of PCR primers

Open boxes and lines designate the gene exons and introns, respectively. The oligonucleotide primers used for PCR (sequences given in the text) are shown above the region of p53 to which they are complementary. The five blocks of amino acids which have been shown to be highly conserved evolutionarily (60) are shown below the corresponding region of the gene.

AOG 9+GC: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGGGATCCTCACCCATCTACAGTCCCCCTT

AOG 10: GCCGGAATTCCTCAGGGCAACTGACCGTGCA

The underlined sequences are specific to the p53 gene and thus anneal to denatured genomic DNA. The remaining bases contain either an Eco RI or Bam HI restriction enzyme site to facilitate cloning of the fragment, and one of each primer pair contains a 40 bp "GC Clamp" (see DGGE, below). These 5' tails are incorporated into the resulting PCR product and are therefore synthetically added to the ends of the p53 fragment from each patient. PCR was performed in a 100 ul reaction containing 1 ug of genomic DNA, 50 pmoles of each primer, 125 uM of each dNTP, 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl pH 8.0, and 1.5 units of AmpliTaq DNA Polymerase (Perkin Elmer-Cetus). The reactions were then taken through 40 cycles of 94 C for 1 minute (denaturation), 50-60 C (optimized for each primer pair) for 1 minute (annealing), and 72 C for 1 minute (extension) using a TAC-3000 thermal cycler.

Denaturing Gradient Gel Electrophoresis (DGGE)

The resulting p53 PCR products were examined for mutations through the use of GC clamped denaturing gradient gel electrophoresis (52). A gradient maker (Hoeffer Scientific) was used to pour 8% polyacrylamide gels (37.5:1 ratio of acrylamide:bis) which contained an increasing linear gradient of denaturants from top to bottom (100% denaturants = 7M urea and 40% w/v formamide). The melting temperature (T_m) of each fragment was determined empirically by electrophoresis of a wild type fragment through a gel which contained a linear gradient from 0 to 80% denaturants perpendicular to the direction of migration. Optimum time of electrophoresis was determined through sequential loadings of parallel gradient gels that ranged from T_m-15% to T_m+15% denaturants. Five pairs of PCR primers divide the p53 gene into <500 bp fragments which can be analyzed for mutations by DGGE. Following PCR from genomic DNA, the samples were ethanol precipitated and resuspended in 30 ul of a buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and .3M NaCl, denatured at 95 C for 5 minutes and reannealed at 58 C for several hours. Ten ul of each sample was added to 10 ul of orange G loading dye, and the resulting samples were electrophoresed at 160 volts on parallel gradient gels at a constant temperature of 60 C. The resulting ethidium bromide-stained gels were examined for mutations as evidenced by altered eletrophoretic mobility of the mutated

fragment and the presence of heteroduplexes (51).

DNA Sequencing

PCR was used to amplify single stranded DNA (53). Sequencing reactions were performed by the dideoxy chain termination method (54) using a modified T7 DNA polymerase (Sequenase, US Biochemicals) (55) and ^{35}S labeled dATP. The four termination reactions were then heated to 75 C for 2 minutes, loaded on an 8% polyacrylamide gel containing 7M urea, and electrophoresed at constant power (60 Watts, 1500-2000 volts) for 3 to 6 hours. The gel was then dried for 1 hour at 80 degrees C and exposed to XAR-5 film (Kodak) for 36 hours. The sequence for the fragment of the p53 gene defined by the primers was read from the gel and compared with the published data for the p53 gene (42).

Results

Loss of chromosome 17p alleles

RFLP analysis revealed deletion of chromosome 17p DNA sequences (Figure 3) in the tumor specimens from eleven of twenty-two (50%) medulloblastoma patients. Of these eleven patients, all demonstrated losses of marker 144-D6 in the distal 17p13.3 subregion and eight also demonstrated losses in the more proximal 17p12 subregion (Figure 4). At least three markers were informative for each of the eleven patients that did not demonstrate loss of heterozygosity for 17p sequences. These markers included 144-D6 which was informative and preserved in all eleven of these patients. Four of the twenty-two patients deleted an allele detected by the human p53 gene sequence (HP53b). There was no detected loss of chromosome 17q sequences.

p53 sequence analysis

Each patient's blood and tumor specimen was examined for point mutations in the p53 gene by PCR amplification followed by DGGE and DNA sequencing. Figure 2 shows the primer pairs used and their location with respect to the intron and exon structure of the human p53 gene. We examined exons 4 through 9 which contain codons 33 through 331 (76% of the open reading frame). This region contains four of the five blocks of highly conserved amino acids. Mutations were detected in only two of the twenty-two (9%) medulloblastoma patients (Figure 5). The mutations were found in exon 7 at codons 242 (Med 15) and codon 248 (Med 17). No germ-line p53 mutations were detected; all blood specimens were found to contain the wild type sequence.

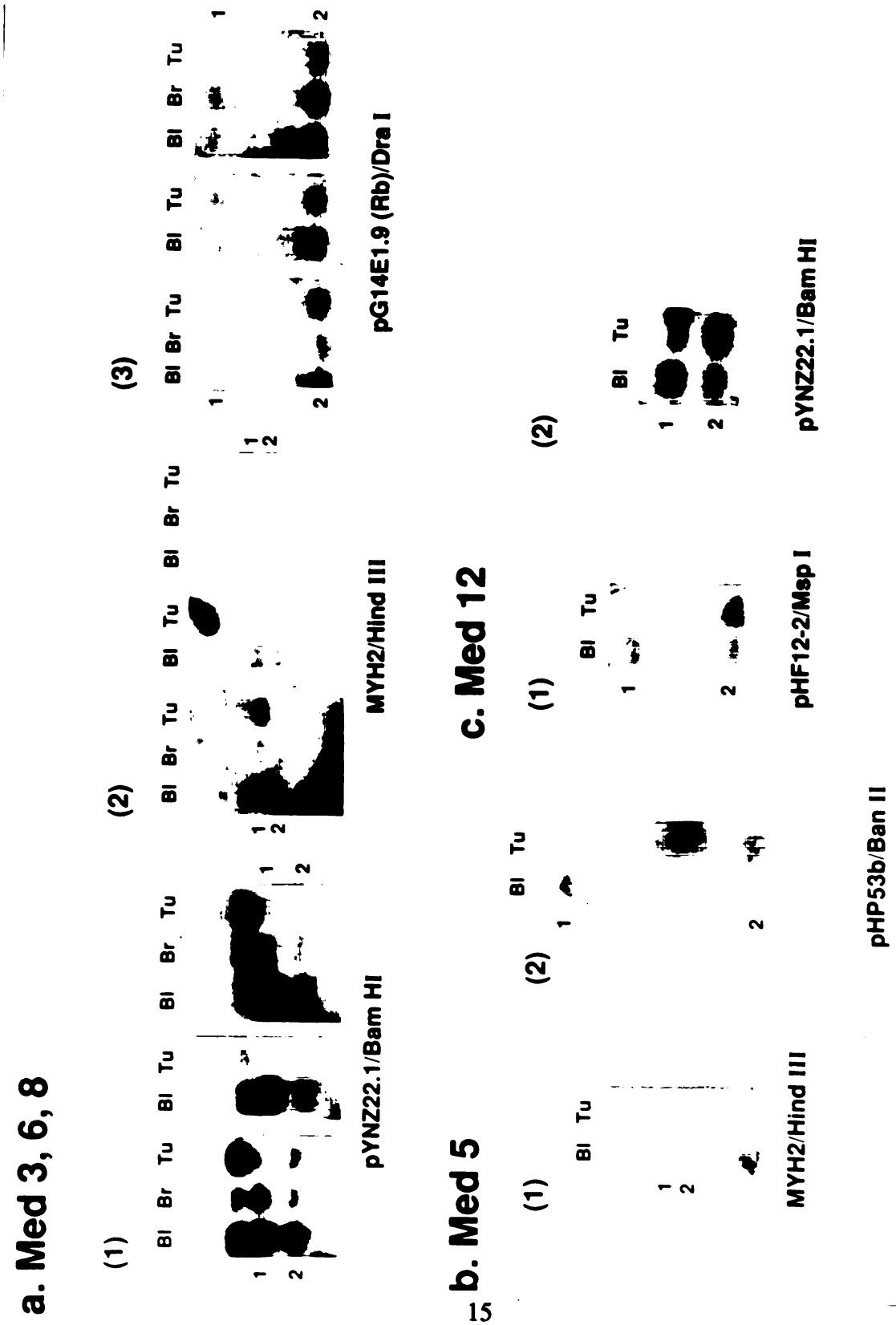
Clinical correlation with genetic data

Tables 3 and 4 divides the twenty two medulloblastoma patients into subgroups based on the RFLP data (no deletion, distal deletion only, proximal and distal deletion), conventional staging criteria ("good" vs "poor" risk), and clinical outcome (alive vs dead or with recurrent disease). Based on the most recently available follow-up data, there appears to be a correlation between clinical outcome and the deletion of RFLP alleles (Table 4). Nine of eleven patients with deletions are dead or have recurrent disease. Examining the ability of conventional criteria to predict outcome for this set of patients, one finds that while the "poor risk" criteria was predictive of a poor clinical outcome,

the absence of these features was not predictive of a favorable outcome. Of the eleven "good risk" patients, five were disease free and six were dead or had recurrent disease. The additional use of the RFLP data improves the ability to predict clinical outcome. Of the eleven good risk patients, 4 of 4 with no deletions are disease free while six of seven with 17p deletions have died or have recurrent disease.

Figure 3 - Allelic losses in medulloblastoma

DNA was extracted from peripheral blood leukocytes (Bl), normal brain adjacent to tumor (Br), and medulloblastoma tumor specimens (Tu). Polymorphic fragments are labeled as "1" or "2" (a) Med-3,6,8. Plates (1) and (2): Loss of chromosome 17 DNA sequences seen for these three tumor specimens taken from the same individual. Plate (3) preservation of alleles in this same patient with the retinoblastoma (Rb) gene. (b) Med-5: Loss of alleles with a chromosome 17 probes including the p53 locus. (c) Med 12: Loss of DNA sequences in the 17p12 subregion but preservation of a more distal 17p13.3 marker.



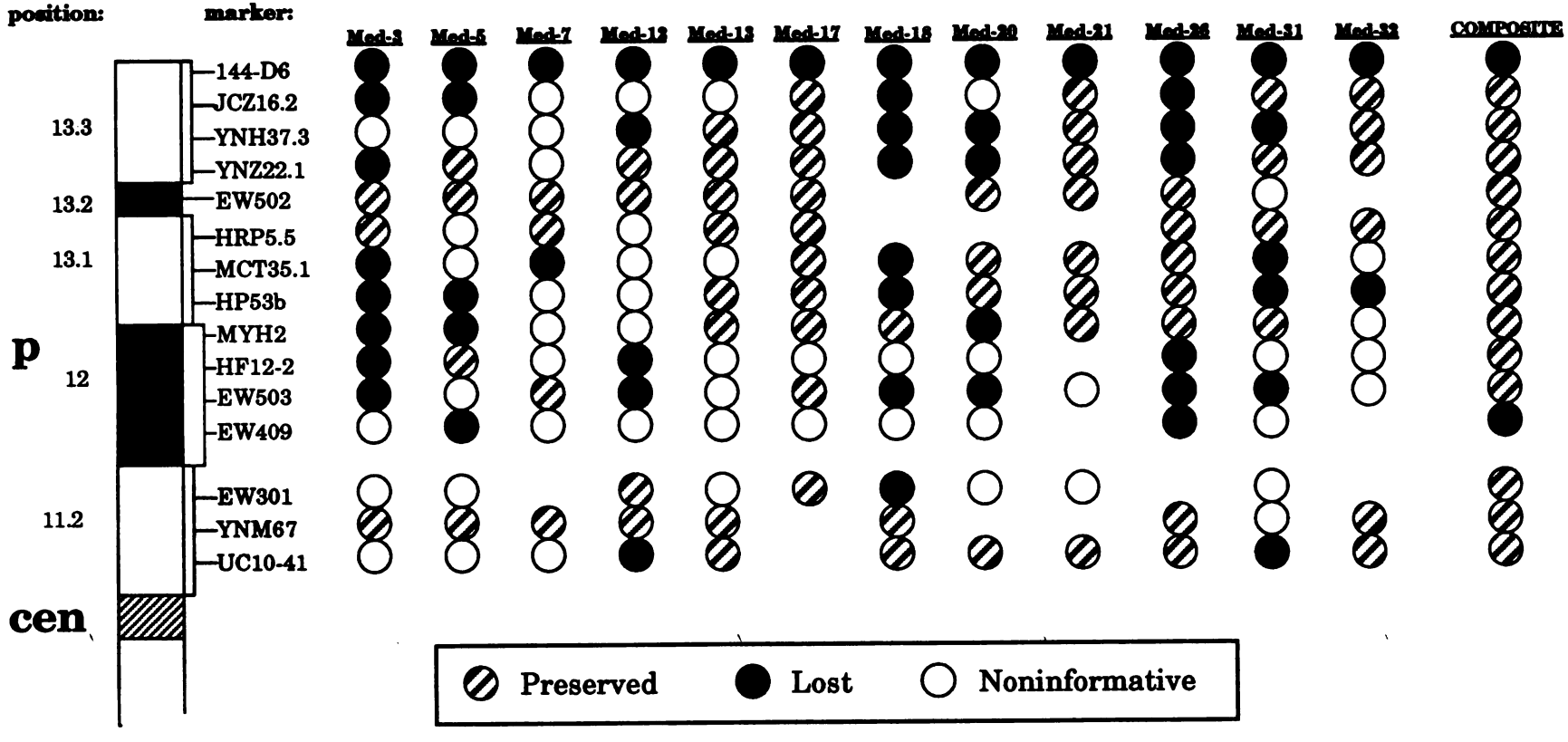


Figure 4 - Deletion map of chromosome 17 for medulloblastoma

The position of chromosome 17p markers on the map are the consensus locations established at the most recent meeting of the Chromosome 17 Human Gene Mapping Committee (57). Patients whose tumors demonstrated loss of chromosome 17p sequences are shown. For each patient, the corresponding marker is scored as either preserved (hatched circle - heterozygous in both the blood and tumor DNA), noninformative (open circle - homozygous in the blood DNA), or lost (black circle - heterozygous in the blood DNA, hemizygous in the tumor DNA). No circle means that the experiment has not been performed yet. The composite was made by finding the smallest common regions of deletion among all patients examined.

Figure 5 - DGGE and sequencing gels showing p53 mutations

(A) Denaturing gradient gel showing new bands (mutant homoduplex + heteroduplexes) observed in the medulloblastoma specimens from two patients (Med-15 and Med-17) in whose tumors p53 mutations were detected. (B) Sequence data for one of two p53 mutations found in medulloblastoma tumor specimens (Med-15). The wild-type sequence found in the patient's blood specimen is shown for comparison. A=adenine; C=cytosine; G=guanine; T=thymine.

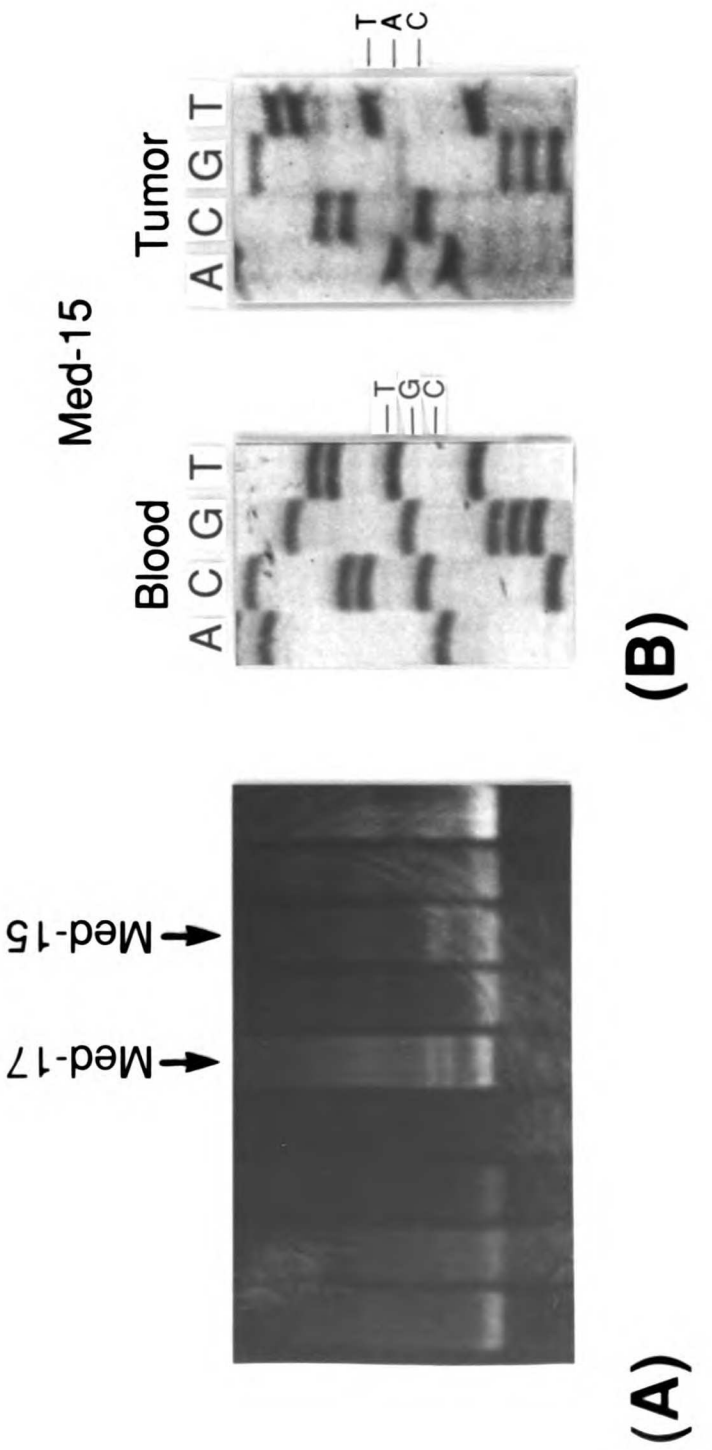


Table 3 - Subgrouping of patients by RFLP data and risk groups

Group A	Group B	Group C
Med-4	Med-13	Med-3,6,8
Med-19	Med-17*	Med-12
Med-22	Med-21	Med-18
Med-24		Med-32

Group D	Group E	Group F
Med-1		Med-5
Med-2		Med-7
Med-10		Med-20
Med-15*		Med-26
Med-16		
Med-28		
Med-29		

Group A - Good risk patients with no deletions
Group B - Good risk patients with distal deletions only
Group C - Good risk patients with distal and proximal deletions
Group D - Poor risk patients with no deletions
Group E - Poor risk patients with distal deletions only
Group F - Poor risk patients with distal and proximal deletions

* Patient's tumor has p53 gene mutation

Table 4 - Clinical outcome vs. RFLP data and risk groups

	No Deletion	Distal Deletion	Proximal and Distal Deletion	TOTAL
Good Risk	A=4 4 disease free 0 dead/recur disease	B=3 1 disease free 2 dead/recur disease*	C=4 0 disease free 4 dead/recur disease	N=11 5 disease free 6 dead/recur disease
Poor Risk	D=7 2 disease free 5 dead/recur disease*	E=0	F=4 1 disease free 3 dead/recur disease	N=11 3 disease free 8 dead/recur disease
TOTAL	N=11 6 disease free 5 dead/recur disease	N=3 1 disease free 2 dead/recur disease	N=8 1 disease free 7 dead/recur disease	N=22 8 disease free 14 dead/recur disease

*patient's tumor has a p53 gene mutation

Discussion

RFLP analysis

Our RFLP analysis demonstrates loss of heterozygosity for chromosome 17p sequences in 50% of the patients examined and thus evidence for the involvement of tumor suppressor genes in the oncogenesis of medulloblastoma. Inspection of the consensus map order of the polymorphic markers used (56, 57) and their pattern of loss in this set of patients (Figure 4) reveals two separate and distinct regions of deletion. All tumors with loss of heterozygosity contained distal 17p13.3 deletions, and a subset also contained more proximal 17p12 deletions with preservation of intervening markers. These findings imply that more than one chromosome 17p tumor suppressor gene may be involved in the oncogenesis of medulloblastoma. A similar pattern of loss has been observed in breast cancer (58) although the more proximal regions of deletion involved 17p13.1 rather than 17p12 markers.

For the remaining 50% of patients that did not demonstrate loss of chromosome 17p sequences, we cannot eliminate the possibility of loss at noninformative loci, small deletions occuring between markers, or point mutations not detectable by RFLP analysis. However, it is interesting to note that marker 144-D6 was always informative; it was lost in all 11 patients that demonstrated chromosome 17p loss and preserved in all 11 patients that failed to do so.

p53 sequence analysis

The first evidence for a tumor suppressor gene on chromosome 17p came from RFLP studies demonstrating deletion of DNA sequences including the p53 locus in neoplasms of the colon (43), breast (58), lung (59), and brain (38). Sequence analysis of the remaining p53 allele in these tumors revealed point mutations in highly conserved regions of the gene (44). It is now widely accepted that p53 is the tumor suppressor gene whose inactivation is detected by 17p allelic loss (60). Mutation to the p53 gene is in fact the most common cancer-related genetic change known at the gene level (61).

Our results suggest that genes on chromosome 17p other than p53 may be involved in the oncogenesis of medulloblastoma. Loss of heterozygosity for the RFLP which maps to the p53 locus was demonstrated in only five of twenty-two patients, and both alleles were clearly preserved in five patients who had lost

proximal and/or distal alleles. Furthermore, we have performed a sequence analysis of the p53 gene for each of the twenty-two patients and have detected only two patients with mutations. Although we did not scrutinize the entire gene sequence, we have sequenced exons 4 through 9 which are known to contain four of the five blocks of highly conserved amino acids (62) where clustering of somatic and germ-line mutations have been previously reported (63,64,65,45). Sequencing of the entire human gene sequence would be required to definitively conclude that these tumors are mutation-free (i.e. to eliminate the possibility of mutations in other parts of the open reading fame, in the regulatory regions of the gene, or in the introns leading to splicing errors). However, given the high frequency of mutation reported in the region we have examined, it seems highly unlikely for our patients to have had p53 mutations in the areas we have not sequenced.

The two p53 gene mutations detected occured at codon 242 (Med-15) and codon 248 (Med-17). Both are contained within exon 7 and domain IV of the five blocks of conserved amino acids, and both were G to A transitions. Transitions from G to A are the most frequent type of p53 mutation seen for brain (75%) and colon (79%) tumors (45). The mutation at codon 242 resulted in an amino acid substitution from cysteine to tyrosine. Substitution at this codon has been previously found, although relatively infrequently compared with other sites (45). The mutation at codon 248 resulted in an amino acid substitution from arginine to glycine. Codon 248 is one of the most frequently reported sites of amino acid substitutions, and has been found to be affected in a wide variety of histologically diverse neoplasms (45). Neither patient demonstrated a loss of heterozygosity at the p53 locus.

Mutation of the p53 gene was nonetheless a relatively infrequent event for this group of medulloblastoma patients whose tumors have clearly demonstrated allelic loss at both proximal (17p12) and distal (17p13.3) loci (66). While we cannot rule out the possibility of mutations in other regions of the p53 gene which have not been sequenced, it was unexpected that so few would be found within the region containing all previously reported mutations. Another p53 sequence analysis of medulloblastoma has also found a paucity of mutations in this gene (67).

It may be the case that distal and proximal deletions of 17p sequences precede mutation of the p53 gene. Indeed p53 mutation has been shown to be a relatively late event in the transformation of adenomatous polyps of the colon to frank carcinomas (32). In an analysis of human astrocytomas, it has been found

that p53 gene mutations are more frequent in higher grade tumors than they are in low grade tumors (68). Our findings of relatively frequent (50%) loss of heterozygosity for 17p but infrequent (9%) p53 mutation argues for the presence of additional or alternate tumor suppressor(s) which may act either separately or in conjunction with p53 in the multi-step process of malignant transformation. Indeed, in breast cancer, overexpression of p53 mRNA has been demonstrated in tumors which contain allelic loss at 17p13.3 but no detectable p53 mutation (58).

Clinical correlation

A correlation between molecular genetic data and clinical status has been previously demonstrated for other tumor types. Studies of colorectal carcinoma have shown that those patients with the greatest number of losses and alterations of chromosomal loci had the worst outcome (32). For breast and ovarian cancer, amplification of the HER-2/*neu* proto-oncogene inversely correlates with survival (69). In neuroblastoma, a childhood tumor with histologic similarities to medulloblastoma (70), allelic deletion of chromosome 1p36 and amplification of the N-*myc* oncogene have been correlated with a poor prognosis (71,72).

It is very encouraging that our genetic data appear to correlate well with clinical outcome for this group of patients (Table 4). Considerable effort has been made to identify risk factors that are predictive of clinical outcome so that treatment may be tailored to minimize toxicity in patients with more favorable disease. To date, the only unequivocal prognostic factor has been the extent of disease at diagnosis as patients with disseminated tumor clearly have a worse prognosis in all studies examining this question (73). Other prognostic factors including the conventional Chang criteria (3) are far more controversial. Indeed, our data demonstrates the relatively poor ability of conventional criteria alone to predict outcome for this set of patients. The genetic data, however, appear to be a much better predictor of clinical outcome and worked best for the subset of patients considered to be good risk, most likely because this constitutes the most homogeneous group studied. It is our hope that these data will help optimize treatment protocols. Our results suggest that "good risk" patients without 17p deletions can be treated more conservatively with surgical resection and radiotherapy, while "good risk" patients with 17p deletions should in fact be treated as "poor risk" patients and should therefore also receive chemotherapy.

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