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Advances in Brief

Gains, Losses, and Amplification of Genomic Material in Rhabdomyosarcoma Analyzed by Comparative Genomic Hybridization[1]

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Abstract

In this study, 10 embryonal and 14 alveolar rhabdomyosarcoma (RMS) tumor samples, including 4 cell lines derived from tumors of the alveolar subtype, were analyzed by comparative genomic hybridization. In the embryonal tumors, the gain of whole or most of various chromosomes, notably chromosomes 2 (60% of cases), 13 (60%), 12 (60%), 8 (60%), 7 (50%), 17 (40%), 18 (40%), and 19 (40%), and the loss of chromosomes 16 (40%), 10 (30%), 15 (20%), and 14 (20%) were found. One case showed evidence of genomic amplification at 12q13–15. In contrast, the alveolar tumors and cell lines showed consistent evidence of genomic amplification, with multiple amplicons in some cases. The amplicons were localized to 12q13–15 (50%), 2p24 (36%), 13q14 (14%), 13q32 (14%), 1q36 (14%), 1q21 (7%), and 8q13–21 (7%). Four cases had additional copies of chromosome 17 or 17q. These changes were in addition to the presence of fusion gene transcripts that are associated with translocations specific to alveolar RMS. The results show that distinct patterns of primarily gains of specific chromosomal material are associated with the embryonal subtype of RMS, and that genomic amplification seems to play an important role in the alveolar subtype. Notably, these distinct changes predominantly involved chromosomes 2, 12, and 13 in both subtypes.

Introduction

RMS[3] is the most common pediatric soft tissue sarcoma and accounts for 4–8% of all malignancies in children less than 15 years old (1). It also occurs in adults, but less frequently, and is rarely seen in persons over the age of 45. Overall, it is the second most common soft tissue sarcoma and is thought to arise from primitive mesenchymal cells that display varying degrees of differentiation towards skeletal muscle (1). RMS-E shows a progressive decline in incidence after 3 years of age, whereas RMS-A shows a bimodal age distribution, peaking at ages 3 and 15 years. RMS-E generally responds well to chemotherapy, in contrast to RMS-A, which requires quite distinct patient management. These differences are reflected at the cytogenetic and molecular level with a loss of heterozygosity at 11p reported to be associated with RMS-E (2), and specific translocations and fusion transcripts associated with RMS-A (3, 4). A large proportion of RMSs (20–60%; Ref. 5) pose significant diagnostic problems, both in terms of distinction from other small round-cell tumors (such as neuroblastomas and Ewing's tumors) and in terms of defining their subtype as either RMS-E or RMS-A (6). Here we have taken a group of tumors that has been well characterized. These tumors included cases that had been studied previously for the expression of genes involved in myogenic differentiation (7) and cases that have been examined for the presence of fusion genes associated with RMS-A; namely, *PAX3-FKHR* and *PAX7-FKHR*, associated with t(2;13)(q35;q14) and t(1;13)(p36;q14), respectively (3, 4). These tumors have been analyzed using the approach of CGH (8) to identify consistent (implying significant) gains and losses of chromosomal material and regions of genomic amplification.

Materials and Methods

Tumor Samples and Cell Lines. Clinical data on the primary RMS samples studied are summarized in Table 1. Cases STS440, STSJP1, and STSJP2 were originally diagnosed as RMS-E, but upon further histological analysis and/or molecular analysis, the classification was changed to RMS-A. The histology of STS251 was uncertain but was clarified after molecular examination. The RMS-E cases STS93, STS238, STS246, STS247, STS248, and STS249 were described previously by Clark *et al.* (7), and the RMS-A case STSSH was analyzed by interphase FISH (9). Cases STS440 and STSJP1 have been described previously (10), as have the cell lines RMS (11), Rh18, Rh28, and Rh30 (12).

Cytogenetic Analysis. Tissue disaggregation, cell culture, and harvesting for metaphase spreads were carried out using standard procedures.

Interphase FISH Analysis. Interphase FISH analysis to detect disruption of the *FKHR* gene was carried out on cases STS251, STS354, and STSPD2 as described previously (9). Cosmid probes spanning exon 1 (cosmid 1) and exons 2 and 3 (cosmid 5) of the *FKHR* gene were used in this analysis (13).

RT-PCR Analysis. RT-PCR analysis for the presence of the *PAX3-FKHR* or *PAX7-FKHR* fusion transcript was carried out as described previously (10).

CGH and Digital Image Analysis. CGH, the capture of digital images, and their analyses were carried out as described previously (10, 14). Briefly, 1 μg of normal and tumor DNA (prepared by standard methods) was directly labeled by nick translation with either fluorescein-12-dUTP or rhodamine-12-dUTP (FlouroGreen and FlouroRed, respectively; Amersham International, Amersham, United Kingdom). The reaction was modified to produce DNA fragments 500-2000 bp in size, as assessed on a 1% agarose gel. Each labeled DNA (300 ng) + 15 μg of *Cot*I DNA (Life Technologies, Inc.) was cohybridized to normal denatured metaphases for 72 h at 37°C before washing and mounting in antifade with 0.1 μg/ml 4′, 6-diamidino-2-phenylindole as a counterstain. Images were captured using a cooled charged couple device camera (Photometrics) with SmartCapture software (Digital Scientific, Cambridge, United Kingdom). CGH analysis was carried out using the same software package and the Quips-XL software (Vysis, Inc., Chicago, IL). At least five representative images were fully analyzed, and the results from these were studied separately and were also combined to produce an average fluorescence ratio for each chromosome.

Results

The cytogenetic, interphase FISH and RT-PCR results on RMS-A tumor samples are indicated in Table 2 and are consistent with this subtype. The cell lines RMS, Rh18, Rh28, and Rh30 were all positive for the *PAX3-FKHR* fusion transcript, and all RMS-E tumors were

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[3] The abbreviations used are: RMS, rhabdomyosarcoma; RMS-E, embryonal subtype of RMS; RMS-A, alveolar subtype of RMS; CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; RT-PCR, reverse transcription-PCR.

Table 1 *RMS studied*

Case	Age[a]/Sex	Subtype	Site
STS93[b]	23/M	RMS-E	Testis
STS238[b]	15/M	RMS-E	Paratestis
STS246[b]	11/M	RMS-E	Paratestis
STS247[b]	6/F	RMS-E	Bladder
STS248[b]	2/M	RMS-E	Calf
STS249[b]	5/M	RMS-E	Paratestis
STSAC[c]	19/M	RMS-E	Buttock
STSPD4	2/M	RMS-E	Calf
STSJP6	31/F	RMS-E	Hand
STSJP7	26/M	RMS-E	Buttock
STS251	3/M	RMS-A	Trunk
STS354	17/F	RMS-A	Pelvis
STS440[d]	4/M	RMS-A	Leg
STSJP1[d]	6/F	RMS-A	Forearm
STSJP2	26/M	RMS-A	Buttock
STSJP3	9/M	RMS-A	Leg
STSJP4	28/M	RMS-A	Arm
STSJP5	16/F	RMS-A	Forearm
STSSH[c]	14/F	RMS-A	Inguinal
STSPD2	12/F	RMS-A	Behind eye

[a] Age in years.
[b] Published previously (see Ref. 7).
[c] Published previously (see Ref. 9).
[d] Published previously (see Ref. 10).

negative for both the *PAX3-FKHR* and the *PAX7-FKHR* transcripts. Disruption of the *FKHR* gene was confirmed by interphase FISH for cases STS251, STS354, and STSPD2 and also provided information on the copy number of the markers used (Table 2). An example of a CGH karyogram from each subtype is shown in Fig. 1, and a schematic summary of the results of CGH analysis of the RMS-E and RMS-A samples is shown in Figs. 2*A* and 2*B*, respectively. The consistent type of aberration identified in RMS-E was a gain of whole or most of various chromosomes, notably chromosomes 2 (6 cases), 13 (6 cases), 12 (6 cases), 8 (6 cases), 7 (5 cases), 17 (4 cases), 18 (4 cases), and 19 (4 cases). A loss of chromosome material was observed for chromosomes 16 (4 cases), 10 (3 cases), 15 (2 cases), and 14 (2 cases). One case showed evidence of genomic amplification at 12q13–15. In contrast, the RMS-A samples showed no consistent gains or losses but, with the exception of one case, showed evidence of genomic amplification, with multiple amplicons in some cases. In 10 RMS-A tumors and 4 cell lines, amplicons were localized to 12q13–15 (7 cases), 2p25 (5 cases), 13q14 (2 cases), 13q32 (2 cases), 1q36 (2 cases), 1q21 (1 case), and 8q13–21 (1 case). Additional copies of chromosome 17 and 17q were also found in RMS-A samples (4 cases).

Discussion

RMSs are a heterogeneous group of tumors both clinically and histologically. The CGH results presented on well-characterized cases of the RMS-E and RMS-A subtypes, although unlikely to represent primary events in tumorigenesis, confirm the underlying differences between these two subtypes and are likely to play a role in tumor progression. The RMS-E samples were characterized primarily by consistent gains of whole or parts of chromosomes (Figs. 1 and 2*A*). This was not found in the RMS-A tumors, in which genomic amplification was the predominating feature found (Figs. 1 and 2*B*).

In a study of RMS-E, 13 of 14 cases were associated with the loss of heterozygosity at 11p13 (2) or more recently, the loss of imprinting (15). Published cytogenetic data does not correlate with this loss, and none of the CGH data presented here showed any evidence of the loss of chromosomal material from this region. However, cytogenetic studies have revealed that the majority of RMS-E cases are hyperdiploid, with an increased copy number of chromosomes 2, 7, 8, 12, and 13 (5). Interphase FISH studies have also demonstrated increased copy numbers of chromosomes 8 and 12, using centromere- specific probes (16), and of chromosome 2 in 9 of 10 cases and chromosome 13 in 6 of the 10 cases, using single copy markers (13). In a similar study (9), the RMS-E case STSAC was trisomic for both chromosomes 2 and 13. The CGH results are therefore consistent with these findings. Cytogenetic evidence for genomic amplification in the form of extra chromosomal elements, double minutes, has been reported in a minority of RMS-E cases (5). Also, CGH analysis of a single case described as RMS-E reported an amplicon at 1p32–33 (17). The present study suggests that genomic amplification is associated predominantly with RMS-A and rarely with RMS-E.

The presence of the fusion transcripts associated with the t(2;13)(q35;q14) and the t(1;13)(q36;q14) is increasingly recognized as being pathognomonic for the alveolar subtype, even in the absence of recognizable alveolar histology. In the series studied, the presence or absence of these fusion transcripts was confirmed. Significantly, all but one case of RMS-A showed evidence of genomic amplification, in keeping with the reported presence of double minutes in some RMS-A cases (5). Of the 10 RMS-A cases and 4 cell lines, 7 showed evidence of amplification at 12q13–15, in addition to 1 RMS-E case. This region has been shown to be amplified in RMSs and also in other human sarcomas (18). In the study of Forus *et al.* (18), 98 human sarcomas were investigated for amplification of known genes from this region. *MDM2* was amplified in 9 of the tumors, *SAS* in 10, *GADD153* (*CHOP*) in 4, *Gli* in 2, and *A2MR* in 2, and the 1 case of RMS had amplification of *SAS* and *CHOP* but not *MDM2*. Thus, genes other than *MDM2* may be important in the cases shown in the present study and require further investigation.

Amplification of the *MYCN* oncogene is known in RMS and almost certainly corresponds to the amplicon at 2p24. Our results suggest that the *MYCN* gene is amplified in 5 of the 14 cases (36%) of RMS-A and none of the RMS-E cases. This is consistent with other studies (19,

Table 2 *Cytogenetic and molecular analysis of RMS-A studied*

Case	Cytogenetic data	Interphase FISH	Fusion transcript by RT-PCR
STS251		*FKHR* disrupted 2 copies	*PAX3-FKHR*
STS354	46,XX,t(2;13)(q35;q14)	*FKHR* disrupted 2 copies	*PAX3-FKHR*
STS440	80–90<4n>,XXYY,del(3)(q13),−13,−13,−13,−20,+mar1,+mar2,4–46dmin[cp10]inc[a]	*FKHR* and *PAX7* amplified[a]	*PAX7-FKHR*[a]
STSJP1	ND[b]	*FKHR* and *PAX7* amplified[a]	*PAX7-FKHR*[a]
STSJP2	45,XX,der(7)t(7;?)(q36;?),der(8)t(8;11)(p23;q13),−10,−11,dup(12)(q11–q22),der(13)t(13;?)(p13;?),−14,+der(15)t(15;?)(p13;?),−16,der(18)t(10;18)(q11;q21),+r,+mar	ND	*PAX3-FKHR*
STSJP3	Complex rearrangement involving 13q14. Tetraploid.	ND	*PAX3-FKHR*
STSJP4	ND	ND	*PAX3-FKHR*
STSJP5	86,XXX,−X,−1,+i(1q),−2,t(2;13)(q35;q14),−3,−4,−5,−7,+13,+13,+der(13)t(2;13)(q35;q14),+3mar	ND	*PAX3-FKHR*
STSSH	ND	*FKHR* and *PAX3* disrupted[c]	*PAX3-FKHR*
STSPD2	ND	*FKHR* disrupted 4 copies	*PAX3-FKHR*

[a] Published previously (see Ref. 10).
[b] ND, not determined.
[c] Published previously (see Ref. 9).

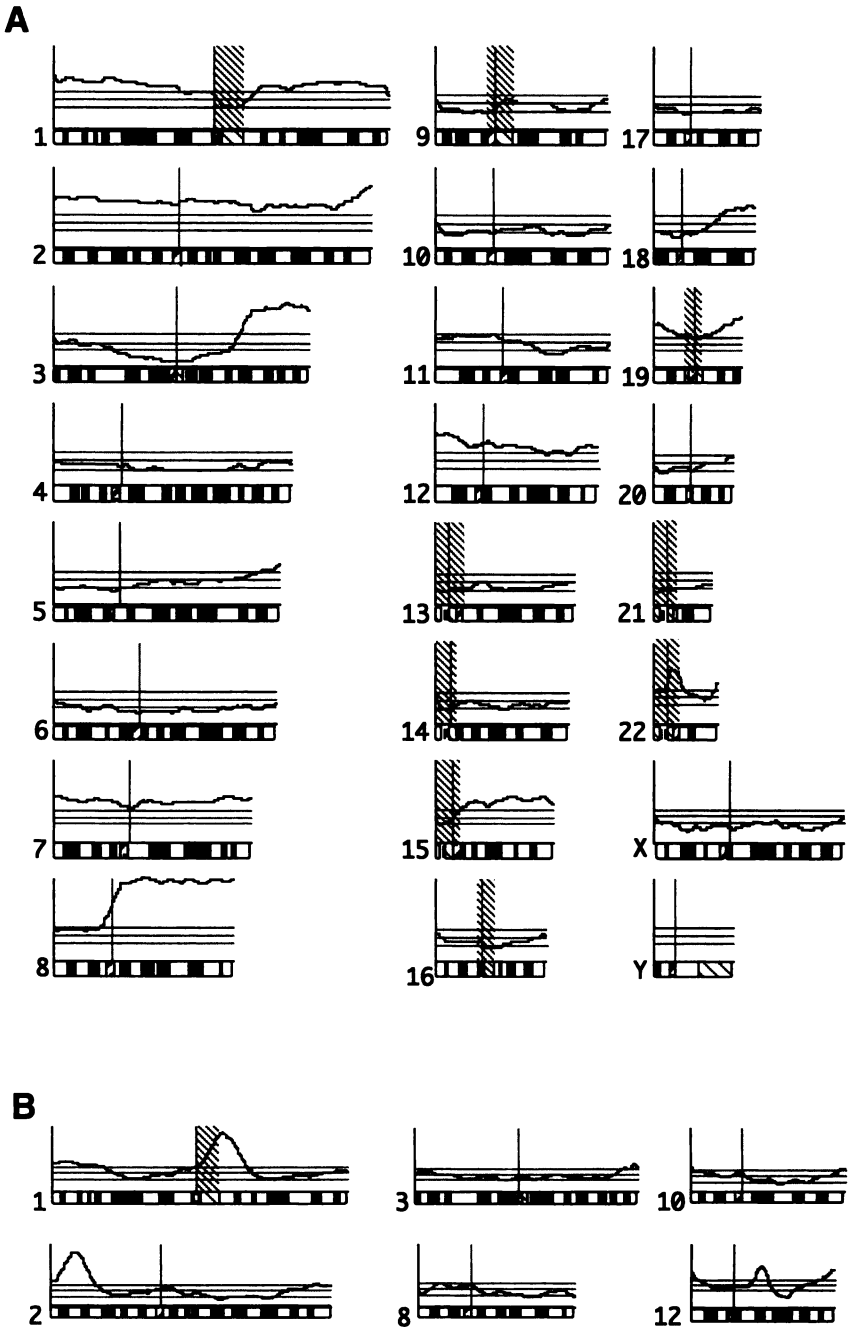

Fig. 1. Average CGH ratio profiles of RMS-E case STSPD4 (*A*) and RMS-A case STSJP3 (*B*). The tumor and normal DNA were labeled with red and green fluorescence, respectively. The average red:green fluorescence intensities were plotted along the length of the chromosome. *Horizontal midline*, a ratio of 1; *lines on either side* of this, a ratio of 0.1 smaller or greater than this. A copy number change is indicated when the profile lies outside these limits. The main heterochromatic regions (*shaded regions*) give unreliable ratios due to suppression with *Cot*1 DNA.

20). Using Southern blots, Driman *et al.* (20) showed evidence of *MYCN* amplification in 3 of 7 alveolar cases (42%) and in none of the embryonal cases. Unlike the situation in neuroblastoma, *MYCN* amplification in RMS has not been shown to correlate with prognosis, possibly because the prognosis of RMS-A is so poor (20).

Amplification at 13q14 and 1p36 has been suggested to correspond to amplification of the fusion gene, associated with the t(1;13)(p36; q14) in cases STS440 and STSJP1, and was the subject of a previous study (10). Approximately 10–20% of RMS-A cases have the *PAX7-FKHR* transcript associated with this translocation, and a recent study (21) has shown that the fusion gene was amplified in 5 of 7 cases with this transcript. Amplification of this fusion gene, and more rarely the *PAX3-FKHR* gene (21), which is associated with the majority of RMS-A cases, may therefore be an important mechanism in oncogenic conversion.

An amplicon at 1q21 was found in one case in the present study. The 1q21–23/4 region has been shown to be amplified in approximately 25% of liposarcomas, leiomyosarcomas, and malignant fibrous histiocytomas (22) but has not been described previously in RMS-A. As the region amplified in this case is small, it may help define the region containing a critical gene. Although an amplicon was present at 8q13–21 in one of the cell lines, amplification at this site has not been reported previously, and its significance to RMS-A requires further investigation. The 13q32 region was amplified in two samples, one of which was reported previously by us (10) and is the subject of further investigation. To our knowledge, amplification of the 13q32 region has not been described in any other tumor type.

The most common amplicons were found on chromosomes 2, 12, and 13 in RMS-A. It is interesting to speculate whether the additional copies of these chromosomes, which were found in RMS-E and also RMS-A cases without amplicons on these chromosomes, might exert

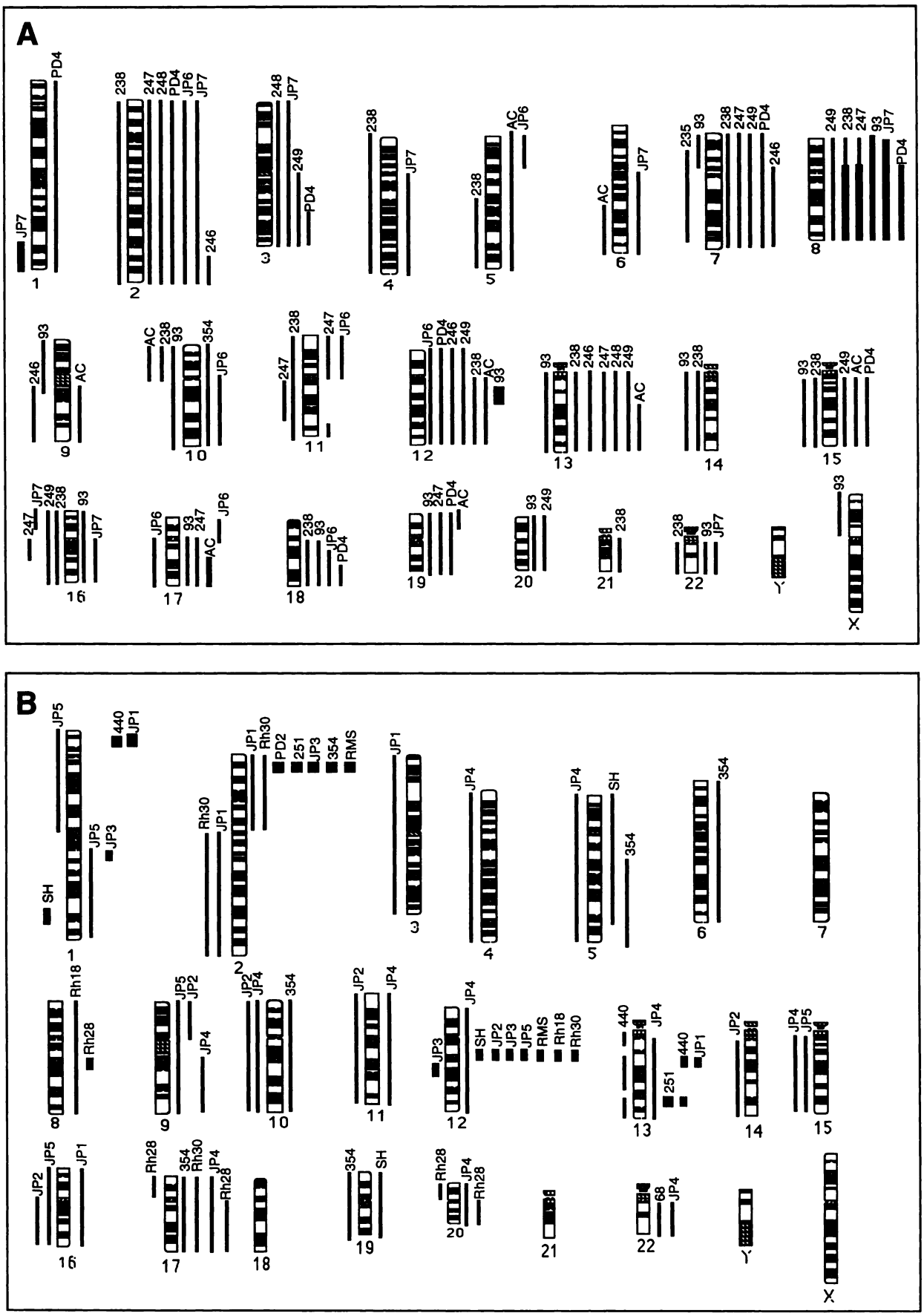

Fig. 2. Summary of the gains and losses of genetic material in the RMS-E (*A*) and RMS-A (*B*) samples analyzed by CGH. *Vertical lines* on the *right side* of a chromosome, a gain of genetic material; *vertical lines* on the *left side* of a chromosome, a loss of genetic material. *Thick lines*, high-level amplification. *Numbers (top of each line)*, case numbers.

a similar effect on amplification by increasing the copy number of specific genes.

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