

Tumor DNA circulating in the plasma might play a role in metastasis. The hypothesis of the genomestasis

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Summary. Background: Clinical and experimental observations suggest that more than one pathway might be involved in the development of metastases. In the present study, we examined the presence of tumor DNA in plasma using an experimental model in which tumor cells were modified with a genome-associated tag. We also investigated whether plasma of tumor-bearing rats had any effect on cultured cells and healthy animals. Methods: Transfected cancer cells (DHD/K12-PROb stably transfected with pCDNA3.1CAT.) were injected subcutaneously into the chest of BD-IX rats. Animals were divided into ten groups according to the time between injection of tumor cells and euthanasia. Prior to euthanasia (2-14 week), blood samples were collected by cardiac puncture. To detect circulating tumor cells and CAT-encoding DNA in plasma, we performed PCR with nested primers. Fifty samples of plasma were chosen at random to supplement the medium of fifty cultures of DHD cells for 10-12 days. PCR for the detection of CAT DNA in cells was performed approximately one to two months later. Four healthy rats received an intraperitoneal injection of plasma from a tumor-bearing rat five times at week for 4 to 6 weeks. Animals were sacrificed and samples of liver, kidney, spleen, omentum, blood and lung were processed by PCR for the detection of CAT DNA. Results: Detection of CAT DNA in plasma was slightly more frequent than in the buffy-coat fraction. All surviving cultures that had been supplemented with plasma were positive at some point for CAT DNA. In all four healthy animals injected with plasma of tumor-bearing rats, the marker gene for CAT was found in extracts of lungs. Conclusion: Our present observation lead us to propose the following hypothesis. Metastases might develop as a result of transfection of susceptible cells in distant target organs with dominant oncogenes that are present in the circulating plasma and are derived from the primary tumor.

Key words: Tumor DNA, Plasma, Metastasis pathways, Colon adenocarcinoma, Rat

Introduction

Most patients with cancer die as a consequence of metastases. It is widely assumed that such metastases arise from seeding of cancer cells from the primary tumor (Mellado et al., 1996). The exact relationship between circulating tumor cells and the development of metastases in specific target organs is unclear. Nonetheless, we do know that the implantation of tumor cells from the peripheral blood is an inefficient process because fewer than 0.1% of circulating tumor cells survive in the circulation (Glavies et al., 1988). Moreover, only few cases have been reported in which tumor cells could be identified in the blood of patients with cancer by sensitive techniques based on the polymerase chain reaction (PCR; Jonas et al., 1996; Mori et al., 1996; Nakamori et al., 1997; Wong et al., 1997; García-Olmo et al., 1999a).

To our knowledge, most PCR-based studies that have focused on the dissemination of tumor cells via the blood of cancer patients have used the cellular fraction of the blood as the source of DNA for amplification by PCR. However, the results of several recent studies indicate that tumor DNA can also be detected in the plasma or serum (Mulcahy et al., 1996; Anker et al., 1997; De Kok et al., 1997; Kopeski et al., 1997).

The detection of free, circulating tumor DNA in the plasma of cancer patients opens up new possibilities for the diagnosis of cancer and prognosis. Most successful studies have been performed using PCR and primers that are specific for different mutations in the *K-ras* oncogene (Anker et al., 1997; De Kok et al., 1997; Kopeski et al., 1997). However, it is unknown whether mutant *K-ras* DNA in the plasma represents extracellular DNA released from a tumor, DNA released from necrotic or apoptotic cells or DNA released as a result of the lysis of fragile, circulating cancer cells. Whatever the mechanism for their presence might be, it is now clear that oncogenes can circulate in the plasma fraction of the blood, and we can now ask whether this phenomenon has potentially important implications in cancer patients.

We have developed an experimental model of cancer using cells with a genome-associated tag (García-Olmo et al., 1999b). In our model, tumors are transplanted in

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rats by subcutaneous injection of DHD cells that have been stably transfected with a plasmid that includes chloramphenicol acetyl transferase-encoding (CAT-encoding) DNA sequences and a neomycin-resistance gene. Our experimental model allows us to detect small numbers of cancer cells in the blood and parenchymous tissues, as well as to examine the presence of tumor DNA in plasma.

The aim of the present study was to examine the presence of free DNA from tumor cells in plasma at different stages of tumor development in rats. We also investigated whether plasma of tumor-bearing rats had any effect on cultured cells and healthy animals.

Materials and methods

Cell culture

The cell line DHD/K12-PROb (also called DHD/K12-TRb; referred to as DHD cells herein) was used.

Generation of genomically tagged DHD-CAT cells by stable transfection and selection

DHD cells were transfected with a plasmid (pCDNA3.1CAT; Invitrogen, Leek, The Netherlands) that included cDNA for a bacterial chloramphenicol acetyl transferase (CAT) under the control of the cytomegalovirus promoter, as well as a neomycin-resistance gene. Transfection was carried out using Lipofectin® (Gibco Laboratories, Grand Island, NY, USA) according to the instructions provided by the manufacturer. Selection for stably transfected cells was initiated three days later by detachment of cells with trypsin and replating in complete medium supplemented with the neomycin analog G-418 (geneticin; Gibco). G-418 was included in the medium at 400 µg/ml for two weeks and routinely included at 200 µg/ml thereafter. Stably transfected cells, whose genome had been permanently tagged with prokaryotic DNA for the CAT gene, were designated DHD-CAT cells.

Animals

Both male and female BD-IX rats were used. Rats in our animal facility are tested annually to ensure that the colony is free of pathogens, such as *Mycoplasma pulmonis*, *Salmonella* sp., Sendai virus, Hantaan virus and Toolan H1 virus. From birth to the end of the experiments, all rats were kept in protective cages with unlimited access to water and standard rat chow (A-04; Panlab, S.L., Barcelona, Spain). A total of 49 animals was used without reference to sex. At the beginning of the experiments, rats were 6 weeks old and weighed 100-150 g.

Implantation of tumors and design of experiments

Tumors were generated in the thoracic region by

unilateral subcutaneous injection of DHD-CAT cells into the right side of the chest. Prior to injection, cultured cells were treated with trypsin, washed, and resuspended in phosphate-buffered saline (PBS). Then 0.25 ml of the suspension, containing 1×10^6 cells, was injected per rat.

After injection, each rat was randomly assigned to one of ten groups. Groups were designated according to the time between the subcutaneous injection of cells and euthanasia, as follows: group 2, sacrificed two weeks after injection (n=5); group 3, sacrificed three weeks after injection (n=5); group 5, sacrificed five weeks after injection (n=5); group 6, sacrificed six weeks after injection (n=5); group 7, sacrificed seven weeks after injection (n=5); group 8, sacrificed eight weeks after injection (n=5); group 9, sacrificed nine weeks after injection (n=6); group 10, sacrificed ten weeks after injection (n=7); group 12, sacrificed twelve weeks after injection (n=2); and group 14, sacrificed fourteen weeks after injection (n=4).

In the case of the last group, we used a separate protocol. From the 11th week after inoculation to the 14th, two to four blood samples were collected weekly from each animal. Blood samples were collected by cardiac puncture under ketamine-xylazine anesthesia (see below for details). This technique was chosen in spite of the associated risks because it was considered to be the best method for obtaining a sufficient volume of sample for amplification by PCR of DNA in the plasma and buffy-coat layer (1 ml). At the end of the 14th week, all the animals in group 14 were sacrificed.

Tumor growth was monitored in all animals and recorded weekly by measurements of greatest diameters with calipers.

Collection of blood samples

At the times indicated by the protocol described above, rats were anesthetized with an intraperitoneal injection of a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg). Then blood (approximately 4 to 4.5 ml per rat) was withdrawn by cardiac puncture. In the case of animals in group 14, 1 ml of blood was collected after each cardiac puncture except at euthanasia, when 4 to 4.5 ml were collected.

All blood samples were collected in tubes with sodium citrate and centrifuged immediately at 2,000g for 15 min. After centrifugation, the plasma and cellular (buffy coat) fractions were carefully collected in separate Eppendorf tubes. Tubes of plasma were centrifuged again for 3 min at 6,000g to ensure the removal of any contaminating cells.

Detection of CAT-encoding DNA sequences in plasma and buffy-coat fractions

To detect CAT-encoding DNA sequences we used a nested-PCR technique that was designed to amplify such sequences. Buffy-coat and plasma samples were processed with a DNA extraction kit (Nucleo Spin

Blood; Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Concentrations of DNA were estimated by measuring absorbance at 260 nm.

Primers were designed on the basis of the published sequence of the CAT gene (Alton and Vapnek, 1979) using MacVector 4.1.4 sequence analysis software (Kodak Scientific Imaging Systems, New Haven, CT, USA) and were manufactured by Pharmacia Biotech (Piscataway, NJ, USA). The protocol used for amplification of CAT DNA, as well as the various parameters of PCR (concentrations of MgCl₂, Taq DNA polymerase, and primers, and the annealing temperature), have been described in a previous report (García-Olmo et al., 1999b)

For an analysis of the sensitivity of the method, one µg of plasmid pcDNA 3.1/CAT, which contained over 1.47x10¹¹ molecules, was serially diluted in PBS and added to rat serum. Under the conditions described in the protocol for the extraction of DNA and nested PCR, we were able to obtain a 237-base pair (237-bp) product that was visible on a 2% agarose gel after electrophoresis and staining with ethidium bromide when we used a starting concentration of 10 copies of the CAT DNA template in 200 ml of rat serum.

Culture of cells with plasma from rats with tumors

Wild-type DHD cells were cultured with samples of rat plasma

Plasma samples were chosen randomly as follows: one from a rat in group 2; five from all the rats in group 7; three from rats in group 8; four from rats in group 10; and two from rats in group 12. Each sample of plasma was added to a different culture and a total of fifteen cultures was studied.

DHD cells were seeded in six-well plates in the standard culture medium

When the cells had reached more than 70% confluence, the medium was replaced by plasma-enriched medium that consisted of an individual sample of plasma diluted (1:2, v/v) in a mixture of DMEM and Ham's F10 (1:1, v/v) and supplemented with 50 µg/ml gentamycin. This medium was replaced with freshly prepared medium every 3 or 4 days for 10 to 12 days. Then cultures were maintained with serial passage in the usual way in culture medium without plasma.

Detection of CAT DNA in cell cultures by PCR

We performed three assays using PCR to monitor the presence of CAT DNA sequences in each culture of wild-type cells that had been cultured with plasma from a tumor-bearing rat. The first PCR was performed 22 to 38 days after addition of rat plasma to the culture medium. The second PCR was performed 12 to 17 days

later and the last PCR was performed 9 days after the second one. Thus, 43 to 64 days elapsed from the start of culture with plasma to the last PCR.

To obtain DNA from cultured cells, we detached cells from plastic plates in the standard manner, washed them twice with PBS, pelleted them and resuspended them in 200 µl of lysis buffer that contained proteinase K. After incubation of the suspension at 55 °C for 30 min and for 10 min at 94 °C, 10 µl of the suspension, containing between 100 and 150 ng of DNA, were used for amplification by PCR, as described above.

Selection by geneticin

To examine transfection with and the possible expression of the neomycin-resistance gene in DHD cells, we added geneticin to selected cultures according to the protocol for the selection of stably transfected cells that we had used for the generation of genomically tagged DHD-CAT cells.

Intraperitoneal injection of plasma from tumor-bearing rats into a small group of healthy rats – a preliminary experiment

Four healthy rats were selected at random. Each received an intraperitoneal injection of 0.25 to 2 ml of plasma from a tumor-bearing rat five times at week. A total of 12 tumor-bearing rats was used. Plasma was obtained as described above and an aliquot of each sample was processed for the PCR assay to examine the presence of CAT DNA sequences. Two animals were injected for four consecutive weeks with plasma from individual animals in which tumor cells had been injected eleven, twelve, thirteen and fourteen weeks previously, respectively. The other two animals were injected for six non-consecutive weeks with plasma from individual rats that had been inoculated with tumor cells one, two, three, nine, ten and eleven weeks previously, respectively. The first two injected animals were sacrificed four weeks after the last injection of plasma and the last two were sacrificed three days later. Blood samples were collected as described above and extracts of samples of liver, kidney, spleen, omentum and lung were also prepared. All samples were processed to PCR for detection of CAT DNA. For assays of blood samples, we used the above-described technique and for assays of tissue extracts, assays were based on a published protocol (García-Olmo et al., 1999b). Samples from two uninjected control animals of similar age, which had been reared under the same conditions, were processed similarly and simultaneously.

Results

We detected the marker gene (for CAT) more frequently in the plasma of rats injected with transfected DHD cells than in the cellular (buffy coat) fraction (in 11.1% and 4.4% of rats, respectively), even several

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weeks after the subcutaneous injection of cells (Table 1). Moreover, differences between frequencies of detection of positive plasma samples and buffy-coat samples were clearer (17.6% and 5.9%, respectively) when blood samples were collected more than once a week from rats from the 11th week after inoculation of tumor cells to the 14th (Table 1).

Cells in four of the cultures died within a few days. In the surviving cultures, we detected the marker gene for CAT by PCR at different times and at different frequencies. The detection of CAT DNA in surviving cells was not associated with prior detection of the marker gene in the added plasma. Indeed, only three of the samples of plasma that were added to cultures were CAT-positive by PCR. Nonetheless, all surviving cultures gave positive results in, at least, one of the three sequential PCR assays that we performed (Table 2).

Table 1. Rates of detection by PCR of CAT-encoding DNA sequences in plasma and cellular (buffy coat) fractions from rats in groups 2 through 12. Animals were divided into groups according to the time in weeks between injection of tumor cells and euthanasia. In group 14, blood samples were collected more than once a week from the 11th week after inoculation to the 14th.

	RATE OF DETECTION IN PLASMA	RATE OF DETECTION IN BUFFY COAT
GROUP 2	0/5	0/5
GROUP 3	0/5	0/5
GROUP 5	0/5	0/5
GROUP 6	2/5	1/5
GROUP 7	1/5	0/5
GROUP 8	2/5	1/5
GROUP 9	0/6	0/6
GROUP 10	0/7	0/7
GROUP 12	0/2	0/2
GROUP 14		
11 th week	1/6	0/6
12 th week	3/8	0/8
13 th week	0/9	1/9
14 th week	2/11	1/11

The first PCR assay of the 11 surviving cultures revealed that nine cultures were CAT-positive (81.8%). The second PCR assay indicated that only two cultures were CAT-positive (20% of ten surviving cultures), while the third indicated that eight cultures were CAT-positive (80% of ten surviving cultures; Table 2).

The plasmid used to transfect DHD cells initially included a gene for neomycin resistance and one of our cultures of cells that had been incubated with the plasma and then supplemented with geneticin after incubation with plasma survived for more than six months (from the time at which geneticin was added to the time of writing).

In all control cultures, wild-type DHD cells grew stably; none was positive for CAT DNA; and all died when geneticin was added to the medium.

In all four healthy animals injected with plasma of tumor-bearing rats, the marker gene for CAT was found in extracts of lungs. Also, we found the marker gene at the following frequencies: 75% (3/4) for plasma; 75% (3/4) for extracts of omentum; 50% (2/4) for buffy-coat samples; 25% (1/4) for extracts of kidney; 25% (1/4) for extracts of spleen; and 25% (1/4) for extracts of liver. In all four animals, at least two of the samples collected were positive. The frequency of detection of positive samples was not related to the timing of sacrifice relative to the last injection of plasma.

Each injected animal had received aliquots of plasma in which the frequency of detection of CAT DNA varied from of 11% to 22%. Similar analyses of samples of parenchymous tissues, plasma and buffy coat from control animals were all negative for CAT DNA.

Discussion

The exact relationship between circulating tumor cells and the development of metastases in specific target organs is unclear.

In our previous studies using this model (García-Olmo et al., 1999b), no circulating cancer cells were

Table 2. Results of attempts at detection by PCR of CAT-encoding DNA sequences and geneticin resistance in DHD cells cultured with plasma from tumor-bearing rats.

ORIGIN OF PLASMA) (GROUP)	POSITIVE SAMPLES OF PLASMA	1 st PCR OF CULTURED CELLS	2 nd PCR OF CULTURED CELLS	3 rd PCR OF CULTURED CELLS	DAYS ELAPSED FROM FROM START OF CULTURE WITH PLASMA TO 3 rd PCR	SURVIVAL AFTER ADDITION OF GENETICIN
2	-	+	+	+	55	-
7	-	+	-	-	55	-
7	-	+	-	-	55	-
8	+	-	-	+	55	-
8	+	+	-	+	55	+
8	-	-	-	+	64	-
10	-	+	-	-	-	-
10	-	+	-	+	43	-
10	-	+	+	+	43	-
10	-	+	-	+	43	-
12	-	+	-	+	55	-
Rate of detection	2/11	9/11	2/10	8/10		

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ever detected in the blood before they were detected in parenchymous tissue. This observation led us to ask what pathway might the tumor cells follow before they reach the target organs. It is theoretically possible that cells are disseminated via the bloodstream in temporary aggregates that might escape detection (Jonas et al., 1996). Our results do not support such a possibility because the amount of blood analyzed from each animal represented almost one-third of the total blood volume of the animal tested and the samples were collected at relatively brief intervals (weekly).

In our above-mentioned study (García-Olmo et al., 1999b), cancer cells were detected in the blood from the 5th week after subcutaneous injection. By that time, the tumors had reached a considerable size (more than 2 cm in diameter; half of the eventual maximum diameter). Thus, it appeared that cancer cells were only detectable in the blood when the tumor was well developed. This finding agrees with other observations in studies of human colorectal cancer (Jonas et al., 1996; Mori et al., 1996; Nakamori et al., 1997; Wong et al., 1997; García-Olmo et al., 1999a) and is also supported by the results of our present study, in which buffy-coat samples were found to be CAT-positive only 6, 8, 13 and 14 weeks after inoculation (Table 1). These results and the low rate of detection of tumor cells in the blood, even when tumors were at advanced stages (Table 1), support the hypothesis that some other pathway, which does not involve the dissemination of cells, might play a major role in the metastatic process.

In the present study, we examined the presence of free DNA from tumor cells in plasma, and we detected CAT DNA in plasma at a similar or slightly higher

frequency than in the buffy coat. These results agree with those in recent studies of human patients that have shown that tumor DNA can be detected in plasma or serum (Mulcahy et al., 1996; Anker et al., 1997; De Kok et al., 1997; Kopreski et al., 1997). What, then, might be the origin of the free tumor DNA that can be found in the blood? Apoptosis or cellular necrosis might yield detectable amounts of extracellular DNA. However, it is also possible that detectable extracellular DNA might be shed from viable tumor cells (Leon et al., 1977).

In this study we tested the ability of tumor DNA in plasma to modify wild-type (without a CAT-encoding DNA sequence) DHD/K12/TRb cells. When the cells were cultured with plasma from tumor-bearing rats, they were transfected with the marker prokaryotic gene and such transfection appeared to be stable (Fig. 1). The variability in the results of the three PCR assays of each culture might be explained by variability in the efficiency of transfection, which is an unknown parameter in our system. However, it is noteworthy that two cultures gave positive results in all three PCRs. Detection of CAT DNA in our cultures was not associated with prior detection of these sequences in the added plasma. Moreover, all culture plates gave positive results in at least one of the PCR assays (Table 2). Thus, the presence of tumor DNA in the plasma might have been more widespread than we were able to detect with our PCR assay.

The transfection of wild-type DHD cells suggests free DNA in the plasma might have appropriate properties for incorporation into the genome of host cell. Moreover, one culture showed neomycin resistance, thus, the genome of some of the cultured DHD cells had not only incorporated a neomycin-resistance gene but also expressed it.

In the early 1980s, transmission of the malignant phenotype from tumor cells to normal cells upon transfection with purified genomic DNA provided direct evidence for the presence of dominant oncogenes in tumor cells (Pulciani et al., 1982a,b). It is now appropriate to ask the following provocative question: could normal cells, for example stem cells, that are located at a distance from a primary tumor become naturally transfected with dominant oncogenes as a result of dissemination of such genes in the plasma? It seems likely that plasma DNA can penetrate cells in an intact organism since parenteral administration of naked DNA that encoded a wild-type p53 anti-oncogene resulted in the suppression of the proliferation of tumor cells (Xu et al., 1997; Zou et al., 1998).

Our present observation that plasma from tumor-bearing rats can transfect cultured cells and the earlier detection of oncogenes in plasma from patients with cancer lead us to propose the following hypothesis. Metastases might develop as a result of transfection of susceptible cells in distant target organs with dominant oncogenes that are present in the circulating plasma and are derived from the primary tumor. We tentatively propose the term "genometastasis" to describe this

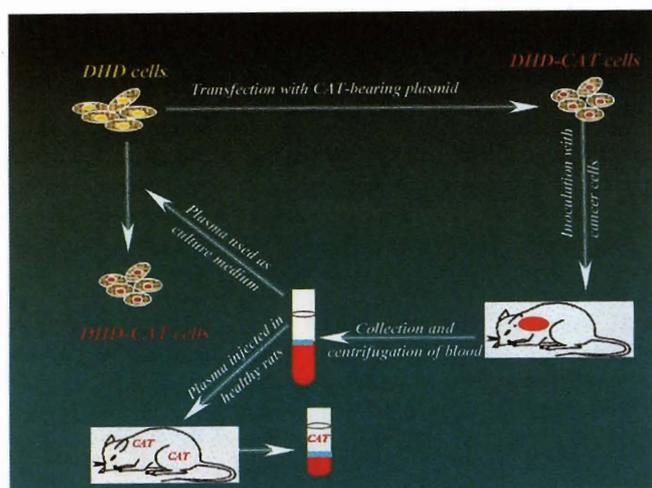


Fig. 1. Schematic representation of CAT-transfection experiments. DHD cells were converted to tagged cells (DHD-CAT cells) in two ways: as a result of direct transfection or as a result of culture with plasma from rats with tumors that had been induced by injection of DHD-CAT cells. Also, in blood and parenchymous tissues of healthy rats that were intraperitoneally injected with plasma of tumor-bearing rats, the marker gene for CAT was detected by PCR.

putative phenomenon.

In a preliminary attempt to examine the validity of our hypothesis, we inoculated four healthy rats intraperitoneally with plasma from tumor-bearing rats. In all four animals, the marker gene for CAT was found in extracts of lungs (Fig. 1). It is noteworthy, in this context that, when rats are inoculated with DHD cells, either subcutaneously or by injection into the wall of the caecum, metastases always develop in the lungs (García-Olmo et al., 1998, 1999b). Also, we found the marker gene for CAT by PCR assays in blood and other tissues so that in all four animals, at least two of the samples collected were positive. These results are obviously preliminary but they do tend to support our hypothesis.

Our hypothesis is also supported by observations that metastatic lesions can be genetically different from the primary tumors from which they originate (Oudejans et al., 1991; Nagel et al., 1995) and that, in mice, the systemic administration of deoxyribonucleases prevents the blood-borne metastasis to the liver of cutaneously transplanted tumor cells (Sugihara et al., 1993).

The evidence in support of our hypothesis is provocative and further investigations are now warranted to test the hypothesis directly.

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Invited Review

Diagnostic and molecular implications of specific chromosomal translocations in mesenchymal tumors

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Summary. In many tumors of mesenchymal origin specific chromosomal translocations are a consistent finding not restricted to malignant tumors. Often the genes behind those translocations have been identified. Rarely, gene activating per se is sufficient to contribute to or cause tumorigenesis whereas in most cases the two genes of the translocation partners both contribute to the formation of a newly arisen fusion gene, thus enabling the detection of resulting chimeric transcripts by highly sensitive techniques. Genes participating in translocations may be affected in more than one tumor entity, such as e.g. *HMGIC* in a variety of benign mesenchymal tumors and *EWS* in a couple of malignant mesenchymal tumors. Certainly, the identification and molecular characterization of specific chromosomal translocations will not only allow for important insights highlighting the process of tumorigenesis but offer also promising new tools to obtain more refined data of diagnostic as well as of prognostic importance.

Key words: Soft tissue tumors, Mesenchymal tumors, Cytogenetics, Chromosomes, Molecular genetic

Specific chromosomal translocations are particularly frequent in tumors of mesenchymal origin

Akin to leukemias cytogenetic analyses of solid tumors are of increasing importance as additional tools helping to establish a correct diagnosis as well as providing prognostically important information (Mitelman et al., 1997). Moreover, they can also guide studies in molecular approaches aimed at establishing the genes involved (Sandberg and Bridge, 1995). This holds particularly true for specific chromosomal translocations (Karakousis et al., 1987) which have been identified in a variety of tumors of mesenchymal origin (Dal Cin and Van den Berghe, 1997; Dei Tos and Dal Cin, 1997; Miller et al., 1997; Fig.1, Tables 1 and 2)

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where they seem to occur more frequently than in epithelial neoplasms. In the latter group only the translocations involving 12q14-15 or 8q12 in pleomorphic adenomas of the salivary gland (Mark et al., 1988; Bullerdiel et al., 1993) and translocations involving 19q13 in follicular thyroid adenomas (Belge et al., 1998) have been identified in a considerable number of cases. Although apparently some more exist such as e.g. witnessed by the t(11;19)(q21;p12-13) in Warthin's tumors (Bullerdiel et al., 1988) and mucoepidermoid carcinomas (Nordkvist et al., 1994) of the salivary gland, in large groups of frequent malignant epithelial tumors cytogenetic studies have failed to detect specific chromosomal translocations akin to those in mesenchymal tumors. The reasons for these differences are largely unknown but it has been hypothesized that they reflect basic differences between both types of tumors. However, the deciphering of the molecular background of the translocations by identification of the genes affected by the chromosomal breaks will not only shed some light on this question but also offer new molecular tools for a more refined classification of the mesenchymal tumors.

We feel that these points deserve a review summarizing main topics of our present knowledge. Nevertheless, such a review cannot address all the interesting and relevant aspects of the theme. Therefore, we want to apologize for any incompleteness due to this problem. If a paper has not been cited this does not allow for the conclusion that the results are less important than those of the papers we have referred to.

Specific chromosomal translocations in mesenchymal tumors are not restricted to malignant neoplasms - the high mobility group protein case

Apparently, many but not all tumors of mesenchymal origin show specific chromosomal translocations. Whereas in the past the occurrence of chromosomal abnormalities has sometimes been interpreted as a sign of malignancy we know today that this is not the case. Many benign tumors of mesenchymal origin including very frequent ones such as e.g. uterine leiomyomas or lipomas can show specific translocations. Interestingly,

Chromosomal translocations in mesenchymal tumors

Table 1. Recurrent non-random translocations in benign and borderline mesenchymal tumors as well as the molecular targets of these translocations.

TUMOR	TYPE OF TRANSLOCATION	GENE(S) INVOLVED	REFERENCES
Angiomyxoma, aggressive dermatofibrosarcoma protuberans, giant cell fibroblastoma	t with 12q14-15 t(17;22)(q22;q13)	<i>HMGIC</i> <i>COL1A1-PDGFB</i>	Kazmierczak et al., 1995 a Pedeutour et al., 1996; Dal Cin et al., 1996; Simon et al., 1997
Hamartoma, lung, pulmonary chondroid ¹	t with 12q14-15	<i>HMGIC</i>	Kazmierczak et al., 1995b, 1996b
Hamartoma, lung, pulmonary chondroid ¹	t with 6p21	<i>HMGI(Y)</i>	Kazmierczak et al., 1996c
Hemangiopericytoma	t with 12q14-15	n.i., <i>HMGIC</i> (?)	Sreekantaiah et al., 1991
Hibernoma	t with 11q13		Dal Cin et al., 1992c
Leiomyoma, uterus	t with 12q14-15	<i>HMGIC</i>	Heim et al., 1988; Schoenmakers et al., 1995; Hennig et al., 1996
Leiomyoma, uterus	t with 6p21	<i>HMGI(Y)</i>	Nilbert et al., 1990; Kazmierczak et al., 1996a
Lipoblastoma	t with 8q11-13	n.i.	Sandberg et al., 1986
Lipoma	t with 12q14-15	<i>HMGIC</i>	Turc-Carel et al., 1986b; Schoenmakers et al., 1995; Ashar et al., 1995
Lipoma	t with 6p21	<i>HMGI(Y)</i>	Mandahl et al., 1988
Polyp, endometrium ¹	t with 12q14-15	<i>HMGIC</i>	Walter et al., 1989; Bol et al., 1996; Dal Cin et al., 1998
Polyp, endometrium ¹	t with 6p21	<i>HMGI(Y)</i>	Dal Cin et al., 1992a; Dal Cin et al., 1998

n.i.: not identified yet.

the genes most frequently affected by chromosomal translocations in benign mesenchymal tumors are the genes encoding the high mobility group proteins of the *HMGI(Y)* family. There are three proteins from that family: *HMGI* and *HMGI-Y* arise by alternative splicing from the same gene, i.e. *HMGI(Y)*, which has been assigned to 6p21.3. The third protein, *HMGIC*, is encoded by a separate gene, *HMGIC*, mapping to 12q15.

Rearrangements of the *HMGIC* and the *HMGI(Y)* locus occur in a huge variety of mesenchymal tumors (Table 1) also including tumors with epithelial as well as mesenchymal part i.e. pulmonary chondroid hamartomas (PCH) and endometrial polyps. However, in the latter tumors it was clearly shown that the chromosomal

translocations involving the 12q14-15 and the 6p21 region are restricted to the stromal part which in the PCHs was also shown by a molecular assay to be of monoclonal origin. 12q14-15 rearrangements or changes involving *HMGIC*, respectively, have not only been detected in "purely" benign tumors such as e.g. lipomas (Fig. 2) but have also been described in a tumor which due to its local aggressive growth has been classified as a borderline tumor i.e. aggressive angiomyxoma (Kazmierczak et al., 1995a). In hemangiopericytomas

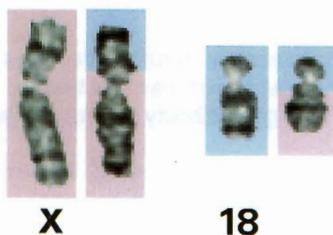


Fig. 1. The translocation $t(X;18)(p11.2;q11.2)$ is a specific translocation found in synovial sarcoma (see Table 2). To indicate the exchange of chromosomal segments more clearly, a pink background is given to the X-chromosome and its segments and a blue background to chromosome 18 and its segments, respectively.

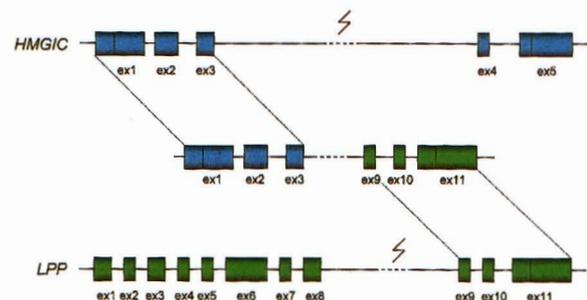


Fig. 2. Schematic representation of the *HMGIC-LPP* fusion gene underlying the translocation $t(3;12)(q27-28;q14-15)$ frequently seen in lipomas as well as in pulmonary chondroid hamartomas. Usually the breakpoint of this translocation occurs in the third intron of *HMGIC* and intron 8 of *LPP*. Thus, the chimeric transcript resulting from this translocation contains exons 1-3 of *HMGIC* (blue) and exons 9-11 of *LPP*.

Chromosomal translocations in mesenchymal tumors

Table 2. Recurrent non-random translocations in malignant mesenchymal tumors, as well as the molecular targets of these translocations.

TUMOR	TYPE OF TRANSLOCATION	GENE(S) INVOLVED	REFERENCES
Alveolar soft tissue sarcoma	t with 17q25	n.i.	
Clear cell sarcoma	t(12;22)(q13;q12)	<i>AFT-1-EWS</i>	Bridge et al., 1991
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	<i>WT1-EWS</i>	Sawyer et al., 1992; Ladanyi and Gerald, 1994; Delattre et al., 1994
Ewing's sarcoma	t(11;22)(q24;q12)	<i>FLI1-EWS</i>	Aurias et al., 1983; Turc-Carel et al., 1983; Giovannini et al., 1994
Ewing's sarcoma	t(7;22)(p22;q12)	<i>ETV1-EWS</i>	Squire et al., 1993; Jeon et al., 1995
Ewing's sarcoma	t(21;22)(q22;q12)	<i>ERG-EWS</i>	Sorensen et al., 1994; Sorensen et al., 1995
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22-q31;q12)	<i>TEC-EWS</i>	Hinrichs et al., 1985; Labelle et al., 1995; Stenman et al., 1995
Liposarcoma, myxoid and round cell	t(12;16)(q13;p11)	<i>CHOP-FUS/TLS</i>	Turc-Carel et al., 1986a; Crozat et al., 1993; Panagopoulos et al., 1995
Liposarcoma, myxoid and round cell	t(12;22)(q13;q12)	<i>CHOP-EWS</i>	Panagopoulos et al., 1996
Rhabdomyosarcoma, alveolar	t(1;13)(p36;q14)	<i>PAX7-FKHR</i>	Douglass et al., 1991; Davis et al., 1994
Rhabdomyosarcoma, alveolar	t(2;13)(q35;q14)	<i>PAX3-FKHR</i>	Seidal et al., 1982; Barr et al., 1993; Galili et al., 1993;
Synovial sarcoma	t(X;18)(p11.2;q11.2)	<i>SYT-SSX1, SYT-SSX2</i>	Limon et al., 1986; Dal Cin et al., 1992b

n.i.: not identified yet.

translocations involving 12q13-15 are a recurrent finding, too, but to the best of our knowledge it has not been shown yet that *HMGIC* is the target of these abnormalities. Furthermore, it should not be neglected that 12q14-15 abnormalities have also been found in about 10-15% of pleomorphic adenomas of the salivary gland indicating that these translocations are very common in mesenchymal tumors but not restricted to them.

As for the biological activity of HMGI(Y) proteins each of them has three DNA-binding domains (DBD) and a short acidic carboxy-terminal part. By their DBDs the HMGI(Y) proteins bind to the minor groove of AT-rich DNA sequences (AT-hooks). Theoretically, they can thus bind to almost any AT-rich DNA sequence longer than 4-5 base pairs. Nevertheless, Yie et al. (1997) have recently been able to show that randomly spaced AT-rich sequences are low affinity binding sites for the proteins, whereas AT-rich sequences which lie on the same strand of the DNA molecule constitute high-affinity binding sites. Based on the example of the beta-interferon promoter the same authors demonstrated how HMGI(Y) appears to recruit and to stabilize complexes of transcriptional activators in the promoter region of that gene thus dictating its transcriptional activity. There is no doubt, however, that the HMGI(Y) proteins are required for the expression of many other eukaryotic genes. Therefore, recently, Hess (1998) has proposed a model for the HMGI(Y)-mediated tumorigenesis. Based on that model the proteins contribute to tumorigenesis by binding to the promoter region of growth controlling genes.

Interestingly, other members of the high mobility group proteins, be they structurally altered or not, have

been proposed to contribute to tumorigenesis as well because the breakpoints of rare cytogenetic subgroups of some of the tumor entities described above coincide with the assignment of some of the genes of the HMGI/2 and HMGI4/17 family members.

Nevertheless, there are other specific chromosomal translocations in benign mesenchymal tumors for which the molecular targets have not been identified yet. In lipoblastomas translocations involving 8q11-12 are a recurrent finding (Sandberg et al., 1986; Fletcher et al., 1993; Panarello et al., 1998) and a group of hibernomas seems to be characterized by translocations involving 11q13 (Dal Cin et al., 1992; Meloni et al., 1994).

Most of the translocations lead to fusion genes detectable as chimeric transcripts

Obviously, so far only for some of the recurrent translocations in tumors of mesenchymal origin the target genes have been identified (Dei Tos and Dal Cin, 1997; Hibshoosh and Lattes, 1997) while other translocations remain to be characterized at the molecular level. Nevertheless, in most of the cases where the molecular background has already been elucidated the translocations lead to fusion genes and accordingly there is only little variation of the translocation partners. Usually, there is only one specific translocation or one type of chimeric transcript, but very rarely tumors with two types of transcripts seem to exist which according to the molecular features can show signs of biphenotypic, such as e.g. muscle and neuroectodermal, differentiation (de Alava et al., 1998).

A well known example for a reciprocal translocation leading to a fusion gene is the t(12;16)(q13;p11). By this

translocation two unrelated genes become fused. The *CHOP* gene maps to 12q13 and encodes for a protein of the CCAAT/ enhancer-binding protein family involved in adipocyte differentiation. The other molecular partner of the translocation is *FUS/TLS*, the protein of which has an RNA-binding domain. Whereas *CHOP* is a member of the C/EBP transcription factor family, i.e. proteins comprising part of the differentiation machinery, the product of the *CHOP-FUS/TLS* fusion presents adipocyte differentiation by directly interfering with C/EBP function (Kuroda et al., 1997; Adelmant et al., 1998). Apparently, this translocation is well compatible with both a myxoid and a round cell morphology allowing for the conclusion that molecularly only one entity can be defined. Interestingly, both *CHOP* and *FUS/TLS* can participate in other specific chromosomal translocations. In the t(12;22)(q13;q12), a variant translocation rarely seen in myxoid and round cell liposarcoma, *CHOP* is fused to *EWS* (Panagopoulos et al., 1996), a gene described in detail later in this review. *FUS/TLS* has been found to be affected by the t(16;21)(p11;q22) characterizing a subtype of AML (Panagopoulos et al., 1995, 1997; Kong et al., 1997; Pereira et al., 1998).

For several other specific translocations the resulting fusion genes have also been identified (Fig.1) but there are still some remaining specific translocations, the molecular background of which has not been elucidated yet. Usually, the existence of chimeric transcripts from the fusion genes allows for a rapid molecular detection by RT-PCR. Using appropriate primers of each one of the translocation partners the presence of these chimeric transcripts can be detected relatively easily with high sensitivity. Thus, RT-PCR offers promising tools for a refined molecular classification of some mesenchymal

tumors (Barr et al., 1995; de Alava et al., 1995; Landanyi, 1995; Kelly et al., 1996). Nevertheless, even if the same genes are affected by an apparently identical translocation, the fusions may be heterogeneous in that the chimeric transcripts contain variant combinations of the exons of the genes involved. This type of heterogeneity may not only affect functional properties of the encoded chimeric transcription factors but is also relevant to the interpretation of molecular diagnostic assays (Antonescu et al., 1998; Meier et al., 1998).

The "molecular promiscuity" of *EWS* and the *HMGI(Y)* genes has a different molecular basis

Of all genes which have so far been identified to be involved in specific translocations, three show a marked molecular promiscuity. *EWS* encodes for a ubiquitously expressed protein (Aman et al., 1996). Akin to *FUS/TLS* the protein contains an RNA-binding domain localized to the carboxy terminal amino acids (Ohno et al., 1994). Interactions of *EWS* with its RNA targets seem to be regulated by phosphorylation by protein kinase (Deloulme et al., 1997) and a region which acts as a transcriptional activator. *EWS* participates in a couple of specific translocations. Apparently, the different types of translocations correspond to distinct histological entities of soft tissue tumors (Fig.3). For example, in dermoplastic small round cell tumors the translocation t(11;22)(p13;q12) is a recurrent translocation. It fuses the amino terminal domain of *EWS* to three of the four zinc fingers of *WT1*, a tumor suppressor gene playing an important role in the development of the kidney and being strongly expressed in the kidneys, gonads and decidua (Ramani and Cowell, 1996). The transcriptional repressor protein is known to bind to heteroduplex DNA

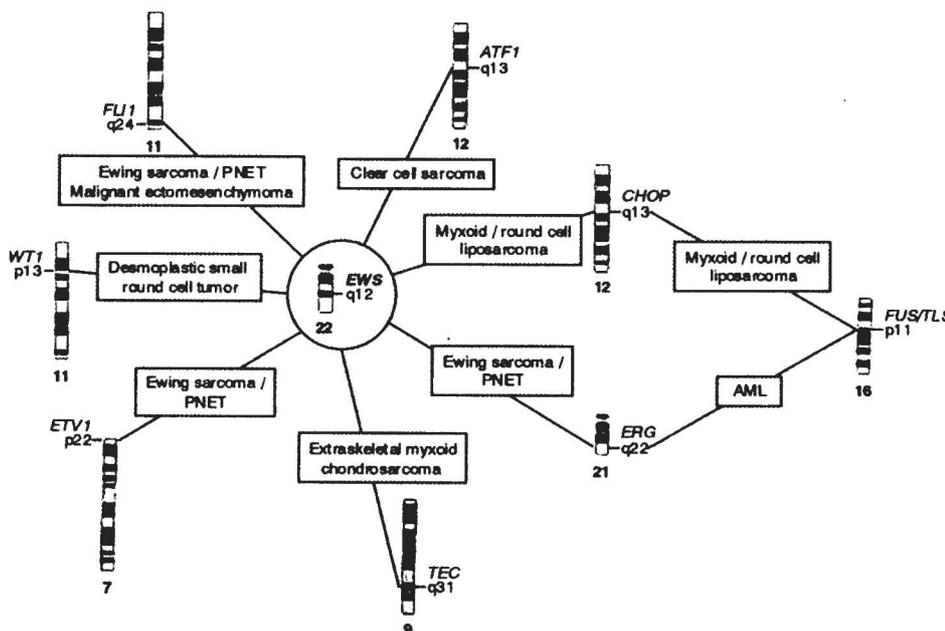


Fig. 3. Diagram illustrating the molecular promiscuity of *EWS*. *EWS* maps to band q12 of chromosome 22 (center, closed circle). By translocations *EWS* becomes fused to other chromosomal bands and genes, respectively. Apparently, the type of translocation clearly correlates with the tumor entity where it occurs. The lines point to translocation breakpoints with other chromosomes and simultaneously, the types of tumors showing the corresponding translocations are indicated. Also, translocations of the *FUS/TLS* are given because there is a link between *EWS* and *FUS/TLS* in that both can fuse to *CHOP* and *ERG*.

(Elser et al., 1997). Due to alternative splicing of the chimeric primary transcript two isoforms of the *EWS/WT1* fusion gene are expressed; only one of which seems to show oncogenic potential by a gain of function (Kim et al., 1998).

Another example of an *EWS* fusion is underlying the t(12;22)(q13;q12) in clear cell sarcomas of soft tissue. That translocation fuses *EWS* to *ATF1* and the resulting fusion protein binds to AFT sites present in cAMP-responsive promoters thereby constitutively activating transcription. Recently, mutational analyses of *EWS/ATF1* have proved to be helpful tools to elucidate the molecular function of the fusion protein in detail (Pan et al., 1998). Interestingly, *EWS* can also fuse to *CHOP* in a variant translocation seen in myxoid and round cell liposarcoma. However, this is not that surprising because *EWS* and *FUS/TLS*, the standard partner of *CHOP* in the common t(12;16)(q13;p11), are housekeeping genes sharing extensive homologies (Aman et al., 1996). Whereas *EWS* has less than 10 translocation partners the situation in the two genes of the *HMG1(Y)* proteins is more complicated. Taking only the simple translocations involving 12q14-15 and only one partner chromosome there are more than 100 different translocations known from the literature and our own cases. For only a few of them the formation of fusion genes has so far been described. A similar finding holds true for *HMG1(Y)*. Moreover the *HMG1(Y)* family differs from the *EWS* case not only by its even higher degree of promiscuity but also by the fact that the type of translocation does not determine the histological type of tumor. Also those translocations which seem to cluster in particular neoplasms, such as e.g. the t(12;14)(q14-15;q24) in uterine leiomyosarcoma or the t(3;12)(q27;q14-15) in lipomas, have been found in other entities as well (Rogalla et al., 1998).

An explanation for this observation is offered by the location of the breakpoints which can be intragenic or in the 5' or 3' vicinity of the genes. Therefore, it seems that even the transcriptional re-activation of both genes may be sufficient to stimulate neoplastic growth (Wanschura et al., 1996). The gene product of fusion genes, however, may have a higher biological activity, as suggested by the result of *in vitro* experiments. In contrast, a deregulated expression of *EWS* per se is apparently not sufficient to endow cells with a tumorigenic potential. The replacement of the *EWS* RNA binding domain by a DNA-binding domain from the fusion partner gene is an event common to all specific translocations which *EWS* participates in. Apparently, by this exchange the resulting proteins gain their oncogenic properties (May et al., 1997).

Looking for specific chromosomal translocations—when, why, and how?

Obviously, during the past decade a lot of important and meaningful advances have been made in the field of tumor cytogenetics which were particularly outstanding

in tumors of mesenchymal origin. The chromosome changes in bone and soft tissue tumors are not only of crucial value in the diagnosis of these lesions but also in the prognostic and therapeutic aspects (Sandberg and Bridge, 1995). Due to their high specificity the chromosomal translocations are of particular importance. Certainly, investigations of a tumor by a conventional karyotype analysis provides the most complete results because not only specific translocations but also all other aberrations visible at the microscopic level can be detected. Therefore, e.g. chromosome abnormalities which occur as secondary changes in addition to the primary specific translocations do not escape attention. For future studies these latter abnormalities should deserve particular interest since they may well be of prognostic significance allowing the prediction of the clinical outcome more precisely. Nevertheless to perform conventional tumor cytogenetics the tumor sample used for the analysis must be suited for cell culturing which has to precede karyotyping. To assure successful karyotyping the tumor sample should be as fresh as possible, kept in sterile saline solution, phosphate-buffered saline, or appropriate cell culture media under sterile conditions prior to setting up the cultures, and most importantly must not be fixed or frozen because cells have to be viable. If shipping to a cytogenetic laboratory is necessary, the samples should arrive there as soon as possible, a period of 24-48 hours being the latest for not significantly reducing the success of the cell cultures. As for the detection of specific chromosomal translocations, fluorescence *in situ* hybridisation (FISH) has become an extremely valuable additional tool which can be applied to slides prepared by conventional cytogenetic methods as well as to interphase cells from uncultured material because it does not depend on dividing cells. Examples of specimens which can be used for FISH are single cells of touch or cytopsin preparations or blood smears, sections of frozen or paraffin-embedded material, and cells or nuclei retrieved from fresh, frozen, or paraffin-embedded samples. Although with these samples the detection of aneuploidies is generally easier than that of translocations, appropriate DNA probes do also allow for analyzing the latter category.

A "pure molecular method" aimed at the detection of chimeric transcripts resulting from specific chromosomal translocations is the polymerase chain reaction (PCR) which was mentioned earlier in this review. Application of the RT-PCR to detect a chimeric transcript due to a translocation requires a full molecular characterization of that translocation and whenever possible samples frozen directly after surgery should be used. However, even paraffin-embedded material can be used to detect chimeric transcripts by RT-PCR.

Specific rearrangements of chromosomes – why do they break where they break?

Whereas for a large number of specific chromo-

somal translocations in mesenchymal tumors the affected genes have been identified, almost nothing is known about factors which may lead to the chromosomal rearrangements. Nevertheless, it is hard to believe that they are no more than the product of random chromosomal aberrations and selection. Rather, it seems very reasonable to postulate the existence of active mechanisms favoring their occurrence. This would also explain the enormously high frequency of some of the specific translocations. A possible mechanism that could account for the exchange of chromosomal segments is related to the existence of transposable elements. These elements are defined as any of a class of diverse DNA segments that can insert into nonhomologous DNA, exit, and relocate in a reaction which is independent of the general recombination function of the host. In several eukaryotic species, transposable genetic elements are known to mediate a variety of genetic phenomena, such as e.g. gene rearrangements. Although it is tempting to assume that the mobile elements account for specific chromosomal translocations in human tumors there is only little direct evidence supporting that hypothesis. Wahbi et al. (1997) were able to show that a t(7;14)(q21;q32) led to the juxtaposition of an immunoglobulin heavy chain locus on chromosome 14 to an endogenous retroviral sequence belonging to a family of transposable genetic elements on chromosome 7. As to translocations involving *EWS* the results of three interesting studies have been published recently. Liu et al. (1997) have described the insertion of a LINE-I DNA mobile element at the genomic breakpoint of a t(11;22)(p13;p12) in a desmoplastic small round cell tumor. Zucman-Rossi et al. (1997) have performed sequence analysis of the entire intron 6 region of *EWS* revealing a very high density of Alu elements. In a rare allele the size of this region is decreased by 50%. Interestingly, this allele has only been observed in an African population characterized by the lowest incidence of Ewing's sarcoma. The breakpoint regions in four myxoid liposarcomas including three with a *CHOP-FUS/TLS* and one with a *CHOP-EWS* fusion gene were investigated by Panagopoulos et al. (1997). In one case of a t(12;16) liposarcoma a sequence similar to the chi bacterial recombination element was found to flank the *FUS/TLS* breakpoint and in the one with the *CHOP-EWS* hybrid gene, the break had occurred close to an Alu element in intron 7 of *EWS*. In all four liposarcomas the breakpoint in intron 1 of *CHOP* was located near an Alu sequence.

The frequent occurrence of specific translocations in mesenchymal tumors compared to epithelial neoplasms is also still waiting for an explanation. So far, only a few specific translocations have been described in epithelial tumors not including the most frequent types, such as e.g. cancer of lung, breast, and colon. Certainly, one might argue that we simply miss them due to extreme complex karyotypic changes not allowing to distinguish between the specific aberration and the "noise" of random cytogenetic deviations. On the other hand, it is unequivocally well documented that malignant epithelial

tumors contain cytogenetically unrelated clones much more often than hematological or mesenchymal neoplasms. Accordingly, a complete carcinoma karyotype is usually thought to be much more complex than extrapolation from the analysis of a few cells may lead us to believe (Pandis et al., 1994). All together, comparison of the patterns of chromosomal and underlying molecular changes in tumors of major embryological cell types demonstrates fundamental differences in genetic mechanisms of tumorigenesis (Rodriguez et al., 1994).

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