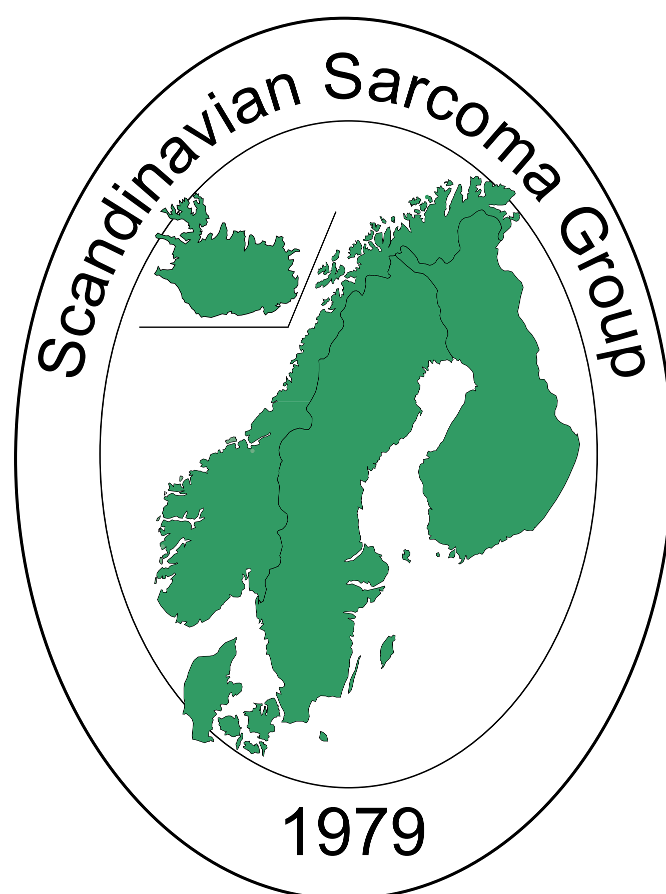
Analysis of Genetic Changes in Musculoskeletal Tumors

3rd edition



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Analysis of Genetic Changes in Musculoskeletal Tumors

1. Intentions

The purpose of this SSG leaflet is to encourage the coordination of genetic analyses of musculoskeletal tumors in the Nordic countries. The intention has not been to define strict guidelines as regards the technical procedures of genetic analysis, but rather to inform about the possibilities and the usefulness of such analyses in the routine work, to give practical advices on how to handle tumor material from the operating room to the laboratory, and to recommend a systematic banking of material to optimize the possibilities to gain as much clinical and basic scientific information as possible from each case. The organization of biobanks is becoming increasingly important since new and refined analytical methods are developed constantly, opening up new avenues of investigations. At the same time, legislation and more or less strong re-commendations on ethical issues, including patient integrity, must be considered when organizing biobanks and planning projects.

2. General background

It is today well established that acquired genetic aberrations, many of which are detectable at the chromosome level, are instrumental in tumor development. Genetic analyses, primarily cytogenetic studies, but in later years also molecular and molecular cytogenetic studies, have been performed systematically on benign and malignant musculoskeletal tumors for almost 20 years. It has been demonstrated that different tumor types have distinct chromosome aberrations, some of which seem to be pathognomonic, and that the consistently involved chromosome bands harbor genes that are essential in tumorigenesis. Identification of specific chromosome aberrations and gene rearrangements (Appendix I) by genetic analysis may be used as important adjunctive diagnostic tools, and there are some recent data that indicate that different chromosome changes, combinations of fusion genes, and types of chimeric transcripts may represent independent prognostic factors. Obviously, there is a great need for continued research along these lines, to find new specific or characteristic genetic chan-ges and to evaluate in larger series the prognostic impact of various genetic parameters.

3. Major techniques for genetic analysis

There is an ever increasing number of techniques to identify and in detail characterize chromosome and gene mutations. These include screening methods and more restricted methods to answer specific questions. Some methods allow the analysis of individual cells and hence give information about intratumor variability, whereas others give information on tissue averages only. Acquired genetic changes in tumors can be investigated at the chromosome level, the genomic (DNA, gene) level and at the expression (RNA, protein) level. Combinations of two or more techniques may be necessary to investigate in depth the characteristics of the tumor cell populations; questions formulated on the basis of findings obtained by screening techniques may be answered using molecular cytogenetic or molecular approaches. Amongst the latter, there is a plethora of techniques that cannot be described individually, suffice it to say that the accomplishments within the HUGO project are steadily increasing the speed by which tumor-associated gene mutations can be identified.

A. ***Cytogenetic analysis by chromosome banding***. Practically all laboratories investigating solid tumors apply short-term culturing (2–14 days). The finding of clonal, acquired chromosome aberrations in 50–70% of the cases of musculoskeletal tumors, including all subtypes, is

usually re- ported. Normal karyotypes could be explained by the presence of sub- microscopic genetic changes, overgrowth by stromal fibroblasts, suboptimal culture conditions and/or inadequate sampling, and are hence non-informative. Viable tumor cells are needed. Samples obtained from surgical specimens, open biopsies, and, for at least some subtypes, fine needle aspiration can be used. Cytogenetic analysis is the superior screening technique; in principle all microscopically detectable chromosome changes are identified independent of initial expectations, and intratumor karyotypic heterogeneity and clonal evolution, if present, can be demonstrated.

B. Polymerase chain reaction (PCR). PCR may be used to investigate suspected gene rearrangements. Because the method is so sensitive, only a small amount of material is required. Since the breakpoints in most recurrent translocations giving rise to fusion genes are scattered over several kilobases, extracted DNA is often not suitable for PCR analysis. In these situations mRNA has to be used in a *reverse transcriptase PCR* (RT-PCR) analysis. Fresh or deep-frozen tissue is needed and access to normal cells from the patient may be useful. Great care has to be taken when using RT-PCR, since the mRNA is easily degraded and has, in some cases, short half-life. The sensitivity and risk of contamination make positive and negative controls important. Knowledge of at least partial gene sequences is required. Positive results are informative but negative results give little information.

C. Southern blotting. This technique may, for example, be used for investigations of loss of heterozygosity (LOH), gene amplification, and rarely for identification of gene fusion at the genomic level. DNA from fresh, frozen, or paraffin embedded tumor tissue (about one million cells per gel track are required) as well as normal cells from the patient are needed. Admixture of normal cells in the tumor sample may make interpretation difficult. Specific questions have to be asked, and it is not a screening technique.

D. Fluorescence in situ hybridization (FISH). FISH (also called molecular cytogenetics) combines cytogenetic and molecular genetic techniques, and has a wide span of resolution, from chromosome level to gene level, depending on the probes used (painting, YAC, BAC, cosmid, centromere specific, telomere specific). In contrast to cytogenetic analysis, FISH is not necessarily dependent on dividing (metaphase) cells since interphase nuclei can be used for investigations of fusion genes and numerical chromosome aberrations. Preparations from tumor tissue or cytological aspirates (short-term cultured cells, imprint or cytospin preparations, cut paraffin embedded tissue) is needed. Often, the probes are selected on the basis of previous cytogenetic findings or a suspected diagnosis. FISH has given unexpected results revealing a higher level of complexity than was expected from the banding karyotype. Interphase FISH allows rapid analysis of many cells, in contrast to methods relying on metaphase cells, which are often limited in number.

The modified FISH techniques, *multicolor FISH* or *spectral karyotyping*, allowing all chromosome pairs to be painted in different colors, represent screening procedures. In one step, the chromosomal origin of the components of multiple interchromosomal rearrangements may be identified. If combined with chromosome banding analysis or conventional FISH analyses, more detailed characterization will be obtained. Chromosome preparations containing dividing tumor cells are required. These techniques, and further modifications using band-specific probe mixtures or multicolor probe sets for simultaneous analysis of several specific aberrations, will probably play a significant role in the future.

E. Comparative genomic hybridization (CGH). CGH allows identification of net genomic imbalances in tumor cells and the chromosomal localization of lost or gained sequences, but

can not detect balanced genomic rearrangements. Fresh, frozen, or paraffin embedded tumor tissue is needed for DNA extraction. Studies of embedded tissues are hampered by decalcification and in these cases fresh tissue is preferred. Substantial admixture of normal tissue decreases the sensitivity of the method. Low copy number gain of sequences can be distinguished from high level amplification. The resolution level of the size of the quantitatively changed sequences is limited to about 3 – 5 megabases, and the sensitivity is lower than for FISH and Southern blotting. CGH, which is a screening method, may also be used in combination with other cytogenetic and molecular genetic techniques and can then yield important additional information.

F. Array techniques. A set of potent hybridization techniques for investigation of a large number of genes simultaneously are being developed. Depending on the nucleic acid sequences or cells spotted on slides or filters, gene mutations and gene expression can be investigated. Literally, information on thousands of genes may be obtained in a single hybridization. Potential problems, when studying up- and down-regulation of genes in a particular tumor type, is what kind of normal tissue should be used as a reference, and how to interprete the extensive information obtained. There is also a development of biostatistical methods that are needed to deal with the large and complex bodies of data that are obtained, as for example at expression profiling of tumors. No doubt, various array techniques will become important research tools, and may probably also be used for diagnostics in a screening fashion. Compared to other molecular methods, relatively large quantities of tissue are required for RNA-based expression studies. Paraffin embedded tissue can be used for the production of tissue arrays containing, for example, a variety of tumor types or tumors of different malignancy grade, which can be used for tests of the expression status of a variety of genes suspected to be involved in tumor development.

4. Genetic analysis of musculoskeletal tumors in the Nordic countries

In the Nordic countries, major centers performing regular clinical analysis and/or basic research on musculoskeletal tumors are located in Bergen, Gothenburg, Helsinki, Lund, Oslo, and Stockholm. Addresses to these centers and names of contact persons can be found in a recent list of SSG members.

5. Clinical use and basic research (and SSG Registry)

The highly specific genetic aberrations of some tumor types and the presence of characteristic aberrations in other tumors may significantly facilitate diagnosis; in some instances the genetic findings alone may be conclusive, but more often the findings will complement clinico-histopathologic investigations to reach a diagnosis. It is important to continue the mapping work, on an as large scale as possible, in order to identify new non-random tumor-associated aberrations and thus further improve the diagnostic power of genetic analysis. Needless to say, the more tumors that are carefully genetically investigated, the better. The availability of such data, together with other data that are regularly included in the SSG Central Registry, will enable investigation of genetic-clinico-pathologic correlations and increase the chances of identifying new prognostic factors.

6. Sampling, transport and storage of tumor material for genetic analyses

Saving tumor material for future investigations when a more specific question can be asked, a new technique becomes available, or new probes or a sufficiently large number of these mostly rare tumors have been collected, is extremely valuable. Material may be saved in many different ways, including paraffin embedding, vital freezing of cultured cells, vital freezing of small tissue pieces,

non-vital deep-freezing of biopsies, freezing of cells in fixative, and saving of fixed unstained chromosome preparations, and smear or imprint preparations. In several cases it may not be possible to save all these types of material, but pathologic, genetic, orthopedic, and oncologic clinics should carefully consider the organization of tumor banks. For practical reasons, the organization of such tumor banks has to be decided at each individual tumor center, with the goal to optimize the conditions as much as possible in relation to the practical and economical realities. A minimum requirement would be to save tumor material in –70/–80 °C. Preferably, the frozen piece should be adjacent to the area(s) selected for histopathologic analysis. It is strongly advisable to freeze also 10 ml peripheral blood (whole blood or pelleted lymphocytes) from the patient, since interpretation of some findings may depend on knowledge on the patient´s genetic constitution.

The requirements on the handling of the material differ depending on the type of analysis to be performed. A general overview of different sampling conditions and some more detailed instructions are given in Appendixes II–IV.

The SSG Tumor Biology Group, April 2000

Appendix I. Characteristic cytogenetic and molecular genetic changes in musculoskeletal tumors

Tumor type	Cytogenetic aberration	Gene aberration
Parosteal osteosarcoma	Ring chromosome(s)	Amplification of 12q sequences
Osteosarcoma (other)	Complex: –13, –3, –10, –15, changes of 1p, 1q, 3p, 3q, 7q, 11p, 17p and 22q	Loss of *RB1* and *TP53*, amplification of *MDM2* and 17p sequences
Extraskeletal (myxoid) chondrosarcoma	t(9;22)(q22;q12) t(9;17)(q22;q11)	*EWS*/*CHN* fusion gene *RBP56*/*CHN* fusion gene
Chondrosarcoma (other)	+7, +20, –10, –6q, –13q, +5, changes of 1q21 and 12q13	–
Chondroma/chondroblastoma/chondromyxoid fibroma	+5, del(6q), changes of 12q13	–
Osteocartlaginous exostosis	del(8)(q24)	Loss of *EXT1*
Giant cell tumor of bone	Telomeric associations	–
Chordoma	–3, –4	–
Ewing sarcoma	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) [t(2;22)(q33;q12)] [t(17;22)(q12;q12)]	*EWS*/*FLI1* fusion gene (~ 90%) *EWS*/*ERG* fusion gene (~5%) *EWS*/*ETV1* fusion gene (<5%) *EWS*/*FEV* fusion gene *EWS*/*EIAF* fusion gene *Prognostic impact of type of fusion transcript?*
Hibernoma	der(11)(q13–21)	Homozygous gene losses in 11q
Lipoblastoma	der(8)(q11–13)	*PLAG1* rearrangements
Ordinary lipoma	der(12)(q13–15) Most common: t(3;12)(q27-28;q14–15)	*HMGIC* rearrangements *LPP*/*HMGIC* fusion gene
Spindle cell/ pleomorphic lipoma	del(13q), del(16q)	–
Atypical lipoma	Ring chromosome(s)	Amplification of genes in 12q
Fibromatosis/desmoid	+8,+20, changes of 5q	Loss of *APC*
Neurilemoma	-22, del(22q)	Loss of *SCH*
Dermatofibrosarcoma protuberans	r(17;22), t(17;22)(q22;q13)	*COL1A1*/*PDGFB* fusion gene
Clear cell sarcoma	t(12;22)(q13;q12)	*EWS*/*ATF1* fusion gene
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWS*/*WT1* fusion gene
Fibrosarcoma:		
Juvenile	+11,+20,+17,+8,t(12;15) (p13;q25–26)	*ETV6*/*NTRK3* fusion gene
Adult	Complex	–
Leiomyosarcoma	Complex	–

Appendix I. Characteristic cytogenetic and molecular genetic changes in musculoskeletal tumors (cont.)

Tumor type	Cytogenetic aberration	Gene aberration
Liposarcoma:		
Well differentiated	Ring chromosome(s)	Amplification of genes in 12q
Myxoid	t(12;16)(q13;p11) t(12;22)(q13;q12)	*FUS*/*CHOP* fusion gene (~90%) *EWS*/*CHOP* fusion gene (~5%)
Pleomorphic	Complex	–
MFH:		
Low grade	Ring chromosome(s)	Amplification of genes in 12q
High grade	Complex: including changes of 1q11–12, and 19p13	–
Neurofibrosarcoma inactivation	Complex: loss Xq26-qter, 11q22-qter, 9p22-pter, 11p13-pter, 17p, 17q11–21	Loss of *NF1*, *CDKN2A*
Rhabdomyosarcoma:		
Alveolar	t(2;13)(q35;q14) t(1;13)(p36;q14)	*PAX3*/*FKHR* fusion gene (~70%) *PAX7*/*FKHR* fusion gene (~5%)
Embryonal	Numerical changes, in particular +2,+8,+20	–
Synovial sarcoma	t(X;18)(p11;q11)	*SYT*/*SSX1, SSX2* or other *SSX* fusion genes *Prognostic impact of SSX1 vs. SSX2?*
Alveolar soft part sarcoma	Changes of 17q25 t(X;17)(p11;q25)	–
Aneurysmal bone cyst	t(16;17)(q22;p13)	–
Inflammatory myofibro-blastic tumor	der(2)(p23)	*ALK*/*TPM3, TPM4*, other fusion genes
Fibrous dysplasia	+2	–
Tenosynovial giant cell tumor	der(1)(p11-13), +5, +7	–

Appendix II

Sampling and transportation of tissue for immediate analysis

If there are any questions or specific requirements, please contact in advance the department to which the sample should be sent.

1. **Samples for cytogenetic and/or metaphase FISH analysis (no freezing or fixation).**

1.1. ***Samples from open biopsies and surgical specimens.***

1.1.1. The tumor tissue should as far as possible be protected from microbial contamination. A piece of viable tissue is removed using sterile instruments. Ideally, tissue adjacent to the sample used for genetic analysis should be taken for histopathologic examination.

1.1.2. The size of the biopsy material should at least be the size of a pea or, preferably, much larger (in particular from adipose tissue tumors) to be able not only to perform the diagnostic analysis but also to save material for future studies. However, no sample is too small not to make an attempt at analysis.

1.1.3. The tumor tissue is transferred to a sterile test tube. For local transportation (up to 1 h) of larger samples no liquid need to be added.

1.1.4. Add sterile physiological NaCl to the tube and fill it up. Close the cap tightly. Liquid is required for samples that are sent and for tiny samples.

1.2. ***Fine needle aspiration samples.***

1.2.1. Request test tubes or small bottles containing any basal tissue culture medium from the department that will perform the analysis.

1.2.2. The tubes or bottles are kept frozen at –18 ^{o}C.

1.2.3. Thaw the medium before use. It should be allowed to reach room temperature.

1.2.4. The aspirate is transferred to the tube or bottle.

1.3. ***Sending of samples.***

1.3.1. The tube or bottle containing the tumor sample is placed in a protective outside tube.

1.3.2. Place the tube together with information about the patient´s name, person identification and, whenever possible, a preliminary diagnosis in an envelope.

1.3.3. Send the sample by express or courier mail. Ideally, the sample should arrive at the genetics department the day after sampling or, at the latest, 2 days after. Remember that no staff is on duty during the weekends.

2. **Samples for molecular genetic analysis**.

2.1. Sampling is performed as above. It may be advantageous to cool the tissue in ice cold physiological saline as fast as possible.

2.2. Fresh tumor tissue is sent as above.

2.3. If the tissue has been frozen it should be sent on dry ice in isolating boxes.

3. **Blood samples from the patient should always accompany the tumor samples**.

3.1. Take (10)–20 ml of peripheral blood in a sodium heparin or EDTA vacutainer or equivalent. Some would prefer citrate rather than heparin.

3.2. If the material is to be sent, place the tube(s) in protective outside tube(s).

3.3 If samples are to be stored before DNA isolation, red blood cells should be removed before freezing.

Appendix III

Storage of tumor material for future investigations.

Several departments (orthopedic, pathologic, genetic, oncologic) may be involved in the analysis of the tumor material. It is important that those involved agree on how material should be saved to enable future investigations. One alternative is that all interested parties receive enough tumor tissue not only to perform routine analyses but also to save material according to their own wishes, needs, and facilities. This should be coordinated at the local plane, and no strict guidelines seem possible at the moment, except for a recommendation to *save as much material as possible*. The expanding national legislation may differ between countries and local ethical committees may have to be addressed. For the analysis of a patient´s genetic constitution (at the molecular level), freezing of peripheral blood cells is mandatory.

1. ***Paraffin embedding of tumors.*** May be used for cutting new sections for additional histopathological stainings, immunohistochemistry, static DNA cytometry, DNA extraction, CGH analysis, and interphase FISH. This saved material should be available from all tumors and should be the responsibility of the pathologists.

2. ***Non-vital freezing of tumor biopsies.*** Pieces of tumor tissue should immediately, or as soon as possible, after surgery be frozen. The tissue should preferably be cut into pieces, and snap frozen in liquid nitrogen (–196 °C). If this is not possible, temporary cooling on ice or in an ordinary freezer (–20 °C can be used. Irrespective of method of cooling or freezing of the material, and even tissue transported at room temperature, should be transferred to a low temperature freezer (– 80 °C), where long-term storage takes place. Suitable for extraction of DNA (Southern blotting, CGH, genomic PCR), RNA (RT-PCR), interphase FISH, and flow DNA cytometry. Ideally, such material should be available from all tumors.

3. ***Vital freezing of tumor tissue.*** Minced tumor tissue may, with or without collagenase treatment, be frozen in liquid nitrogen so as to retain cell viability, i.e., in medium containing bovine calf serum (~20%) and 10% DMSO. Such material can be used for the same purposes as in 2 and for the initiation of tissue cultures and hence also for metaphase analysis. The technique is fairly time consuming and should be optional.

4. ***Vital freezing of tissue cultures.*** Cultured cells may be frozen in liquid nitrogen so as to retain cell viability. The cells can be used for extraction of DNA, RNA, and proteins, cytogenetic and FISH analyses, establishment of permanent cell lines, and xenografting. Freezing is not always possible (cultures used up for routine analyses) or worth while (only stromal fibroblasts growing). Fairly time consuming and should be optional.

5. ***Saving of fixed cells.*** If possible, cultured cells may be saved as cell suspensions in fixative in an ordinary freezer (–20 °C). The cells can be used primarily for making new slides for chromosome banding analysis and metaphase and/or interphase FISH. The responsibility rests with the (cyto)geneticists. Also chromosome and imprint preparations may be saved under desiccation in a freezer for similar purposes.

6. ***Permanent cell lines and xenografts.*** Permanent tumor cell lines and xenografted, serially transplanted tumor cells may be extremely useful as practically unlimited resources of material from individual tumors. All kinds of investigation can be performed and lines are useful as positive controls. A drawback is that they may lose some original (usually not the primary) and acquire new genetic rearrangements. Attempts to establish such cell lines are not always successful and are both time- and labor-consuming.

Appendix IV

A. Usefulness of different types of material for different methods of genetic analysis.

Method	Fresh vital tissue	Vital frozen (–196 ºC) cells/tissue	Frozen tissue (–80 ºC)	Paraffin embedded tissue	Cell sus-pension in fixative	Cell line
Chromosome banding analysis	Yes	Yes	No	No	Yes	Yes
Metaphase FISH	Yes	Yes	No	No	Yes	Yes
Interphase FISH	Yes	Yes	(Yes)	(Yes)	Yes	Yes
Southern blot	Yes	Yes	Yes	(Yes)	Yes	Yes
PCR	Yes	Yes	Yes	(Yes)	Yes	Yes
RT-PCR	Yes	Yes	Yes	No	No	Yes
CGH	Yes	Yes	Yes	Yes	No	Yes
DNA flow cytometry	Yes	Yes	Yes	(Yes)	Yes	Yes
Gene expression array	Yes	Yes	Yes	No	No	Yes

B. Usefulness of material for different types of genetic analysis in relation to the method of banking tumor tissue.

Sample handling	DNA-based based analysis	RNA-based analysis	Protein-based analysis	Metaphase chrom. analysis	Interphase chrom. analysis
Snap freezing in N_2 or immediate cooling, rapid freezing* and frozen transport to lab. Long-term storage at –80 ºC	Excellent but not necessary	Optimal	Optimal	Not possible	Possible
Immediate cooling on ice **, rapid cooled transport to lab. Long-term storage at –80 ºC	Works fine	Possible in most cases	Possible in most cases	Possible before deep freezing but not after	Possible
Rapid transport at room temperature to lab. Long-term storage at –80 ºC	Works fine	Possible for some RNAs only	Variable	Works fine before deep freezing but not after	Possible
Transport at room temperature, no freezing	Possible	Only rarely possible	Not possible	Works fine	Works fine

* Ice box in the operation room and a –20 ºC freezer (for short-term storage, up to 48 hours) in the operation room or its vicinity.

** Ice box in the operation room, transport to freezing facilities within 2 hours.