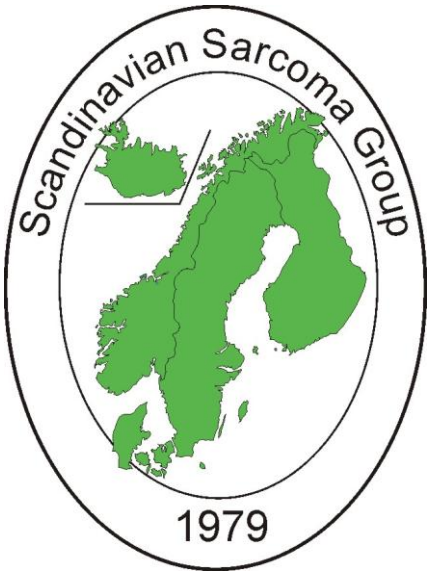
SSG Morphology and Tumorbiology Group
2012

Analysis of Genetic Changes in Musculoskeletal Tumors

4th edition



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Analysis of Genetic Changes in Musculoskeletal Tumors

1. General background

It is well established that acquired genetic aberrations, many of which are detectable at the chromosome level, are instrumental in tumor development. Genetic analyses have been performed systematically on benign and malignant musculoskeletal tumors for 30 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 harbour 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. Obviously, there is a great need for continued research along these lines, to find new specific or characteristic genetic changes and to evaluate in larger series the prognostic impact of various genetic parameters.

2. Major techniques for genetic analysis

There are an ever increasing number of techniques to identify and in detail characterize chromosome and gene mutations. These include screening methods and directed 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.

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 reported. Normal karyotypes could be explained by the presence of submicroscopic 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, core-needle biopsies, and, for at least some subtypes, fine needle aspiration can be used. Cytogenetic analysis has the advantage of identifying balanced as well as unbalanced rearrangements, 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 usually needed, but paraffin blocks can sometime be used, 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. 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, locus-specific, 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) are needed. Often, the probes are selected on the basis of a suspected diagnosis. 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. Chromosome preparations containing dividing tumor cells are required.

D. Array-based methods: High-resolution microarray technologies are today used to screen tumor genomes for copy number gains and losses as well as for global gene expression profiles, methylation patterns, microRNA expression, etc. Companies manufacturing such arrays use slightly different approaches; thus, extraction methods, software needed for evaluation of results, etc vary depending on the platform used. Although DNA or RNA from paraffin-embedded tissue can be used for some purposes, fresh or fresh-frozen tissue is preferable. It should be noted that DNA-based approaches ("array CGH") do not detect balanced chromosomal rearrangements, i.e., most rearrangements resulting in fusion genes will go undetected. It should also be emphasized that the quality of the extracted RNA is of much greater importance for a global gene expression analysis than for an RT-PCR analysis for a putative fusion gene.

E. Next-generation sequencing: The last few years, a number of different technologies allowing the sequencing in one experiment of entire genomes, all transcribed sequences (the "transcriptome"), all exons from coding genes (the "exome"), or selected (captured) sequences from a large number of genes or chromosomal regions have become available. Not only do such analyses promise to detect all possible sequence changes, but also copy number alterations can be visualized and, if RNA is used, an expression profiling can be achieved. The costs for such experiments are still high, and the technology is presently mainly used for research purposes. It is, however, reasonable to assume that next-generation sequencing will replace several other methods in the next 5-10 years.

3. 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.

4. 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, orthopaedic, 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 centre, 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 ^{o}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 Morphology / Tumor Biology Group, March 2012

Appendix I

Characteristic cytogenetic and molecular genetic changes in musculoskeletal tumors

Tumor type	**Characteristic chromosome aberrations**	**Characteristic molecular genetic findings**
Bone tumors		
Aneurysmal bone cyst	t(16;17)(q22;p13) and other rearrangements of 17p13	*CDH11/USP6* fusion gene and other fusion genes involving *USP6*
Chondroma/chondroblas-toma/chondromyxoid fibroma	rearrangements of chromosome 6	-
Chondrosarcoma, conventional	complex	-
Chondrosarcoma, mesenchymal	-	*HEY1/NCOA2* fusion gene
Chordoma	complex	-
Desmoplastic fibroblastoma	rearrangement of 11q12-13	activation of *FOSL1*
Ewing sarcoma	t(11;22)(q24;q12) t(21;22)(q22;q12) and other rearrangements affecting 22q12 or 16p11	*EWSR1/FLI1* fusion gene (~ 90%) *EWSR1/ERG* fusion gene (~5%) and other fusion genes involving *EWSR1* or *FUS*
Giant cell tumor of bone	telomeric associations	-
Osteochondroma	del(8)(q24)	loss of *EXT1* or loss of *EXT2*
Osteosarcoma, high grade	complex	complex
Osteosarcoma, parosteal	ring chromosome(s)	amplification of 12q sequences, including *MDM2*
Undifferentiated sarcoma, pleomorphic	complex	-

Soft tissue tumors

Alveolar soft part sarcoma	t(X;17)(p11;q25)	*ASPSCR1/TFE3* fusion gene
Angiosarcoma	complex	amplification of *MYC*
Chondrosarcoma, extraskeletal myxoid	t(9;22)(q22;q12) t(9;17)(q22;q11) and other rearrangements involving 9q22	*EWSR1/NR4A3* fusion gene *TAF15/NR4A3* fusion gene and other fusion genes involving *NR4A3*
Clear cell sarcoma	t(12;22)(q13;q12) t(2;22)(q33;q12)	*EWSR1/ATF1* fusion gene *EWSR1/CREB1* fusion gene
Dermatofibrosarcoma protuberans	r(17;22), t(17;22)(q22;q13)	*COL1A1/PDGFB* fusion gene
Desmoid tumor	+8,+20, del(5q)	loss of *APC*
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWSR1/WT1* fusion gene *EWSR1/ERG* fusion gene
Epithelioid hemangioendothelioma	t(1;3)(p36;q25)	*WWTR1/CAMTA1* fusion gene
Epithelioid sarcoma	complex	loss of *SMARCB1*
Fibrosarcoma, infantile	+11,+20,+17,+8,t(12;15)(p13;q25-26)	*ETV6/NTRK3* fusion gene
Hemosiderotic fibrolipomatous tumor	t(1;10)(p22;q24) der(3p)	Rearrangement of *TGFBR3* Amplification of *VGLL3*
Hibernoma	der(11)(q13-21)	loss of *MEN1* and *AIP*
Inflammatory myofibro-blastic tumor	t(2;17)(p23;q23) and other rearrangements of 2p23	*CTLC/ALK* fusion gene and other fusion genes involving *ALK*
Leiomyosarcoma	complex	-
Lipoblastoma	der(8)(q11-13)	*PLAG1* rearrangements
Lipoma, atypical	ring chromosome(s) giant marker(s)	amplification of genes in 12q, incl *MDM2*, *CDK4* and *HMGA2*
Lipoma, chondroid	t(11;16)(q13;p13)	*C11 or f95/MKL2* fusion gene

Lipoma, conventional	t(3;12)(q27-28;q14-15) and other rearrangements affecting 12q13-15	*LPP/HMGA2* fusion gene and other rearrangements of *HMGA2*
Lipoma, spindle cell	del(13q), del(16q)	-
Liposarcoma, myxoid/round cell	t(12;16)(q13;p11) t(12;22)(q13;q12)	*FUS/DDIT3* fusion gene *EWSR1/DDIT3* fusion gene
Liposarcoma, pleomorphic	complex	-
Liposarcoma, well-differentiated and de-differentiated	ring chromosome(s) giant marker(s)	amplification of genes in 12q, including *MDM2*, *CDK4* and *HMGA2*
Low grade fibromyxoid sarcoma	t(7;16)(q33;p11) t(11;16)(p11;p11)	*FUS/CREB3L2* fusion gene *FUS/CREB3L1* fusion gene
Malignant peripheral nerve sheath tumor	complex	loss of *NF1* and *CDKN2A*
Myoepithelial tumor	t(2;22)(q33;q12) and other rearrangements of 22q12	*EWSR1/CREB1* fusion gene and other fusion genes involving *EWSR1*
Myxofibrosarcoma	complex	-
Myxoinflammatory fibroblastic sarcoma	t(1;10)(p22;q24) der(3p)	Rearrangement of *TGFBR3* Amplification of *VGLL3*
Nodular fasciitis	t(17;22)(p13;q13)	*MYH9/USP6* fusion gene
Rhabdoid tumor, extrarenal	der(22q)	loss of *SMARCB1*
Rhabdomyosarcoma, alveolar	t(2;13)(q35;q14) t(1;13)(p36;q14)	*PAX3/FOXO1* fusion gene *PAX7/FOXO1* fusion gene and other fusion genes involving *PAX3*
Rhabdomyosarcoma, embryonal	numerical changes, in particular +2,+8,+20	-
Rhabdomyosarcoma, pleomorphic	complex	-

Schwannoma	-22, del(22q)	loss of *NF2*
Soft tissue angiofibroma	t(5;8)(p15;q13)	*AHRR/NCOA2* fusion gene
Synovial sarcoma	t(X;18)(p11;q11)	*SS18/SSX1*,*SS18/SSX2* or *SS18/SSX4* fusion genes
Tenosynovial giant cell tumor	t(1;2)(p13;q37) and other rearrangements of 1p13,+5,+7	*COL6A3/CSF1* fusion gene and other rearrangements of *CSF1*
Undifferentiated sarcoma, pleomorphic	complex	-
Undifferentiated sarcoma, round cell	t(4;19)(q35;q13)	*CIC/DUX4* fusion gene and various *EWSR1* rearrangements

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 needs 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 usually 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 (orthopaedic, 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 should be frozen. The tissue should preferably be cut into pieces, and snap-frozen in liquid nitrogen (-196 ^{o}C). If this is not possible, temporary cooling on ice or in an ordinary freezer (-20 ^{o}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 ^{o}C), where long-term storage takes place. Suitable for extraction of DNA, RNA, 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 worthwhile (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 ^{o}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 °C) cells/tissue	Frozen tissue (-80 °C)	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
PCR	Yes	Yes	Yes	(Yes)	Yes	Yes
RT-PCR	Yes	Yes	Yes	(Yes)	No	Yes
Array-based analysis	Yes	Yes	Yes	(Yes)*	No	Yes
Global gene expression array	Yes	Yes	Yes	No	No	Yes
Next-generation sequencing	Yes	(Yes)	Yes	(Yes)*	No	Yes
DNA flow cytometry	Yes	Yes	Yes	(Yes)	Yes	Yes

* Problems with decalcification in bone tumors.

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

SAMPLE HANDLING	DNA-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 °C	Excellent but not necessary	Optimal	Optimal	Not possible	Possible
Immediate cooling on ice **, rapid cooled transport to lab. Long-term storage at –80 °C	Works fine	Works fine	Possible in most cases	Possible before deep freezing but not after	Possible
Rapid transport at room temperature to lab. Long-term storage at –80 °C	Works fine	Variable	Variable	Works fine before deep freezing but not after	Possible
Transport at room temperature, no freezing	Variable	Variable	Not recom-mended	Works fine	Works fine

* Ice box in the operation room and a –20 °C 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.