PROGNOSTIC IMPACT OF MICRO-RNA IN SOFT TISSUE SARCOMA

This project is based on a translational research collaboration between UNN, UiT and University Hospital of Arkhangelsk

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1. **Summary**

The project aims to explore the role of a variety of essential microRNAs in soft tissue sarcoma (STS) as predictors for treatment response, metastasis, survival and treatment strategies. Together with demographic, clinical and pathological data selected microRNAs will be investigated within subgroups of STS. The costs of the research protocol is estimated to be **116.000,- Nkr**.

The research is based on tissue micro array (TMA) from resected primary tumors of 800 operated STS patients, 50% from hospitals in Helse Nord and 50% from Arkhangelsk, Russia.

The expression of more than 50 essential molecular markers belonging to angiogenesis, immune function, and the processes of epithelial-to-mesenchimal transformation (EMT) of mesenchymal cancer cells are investigated by immunohistochemistry.

We have recently established new methods for isolation and assessment of microRNAs from the tumor specimens. MicroRNA is a new and abundant class of RNA regulatory genes that confer a novel layer of genetic regulation in cells. They are endogenous, highly conserved, small RNAs that silence gene expression by binding to targets mRNAs. Preliminary results from our group indicate significant prognostic values of the micro-RNAs Hsa-let-7a and hsa-miR-155 in the STS tumors.

Sarcomas represent a large heterogeneous group of mesoderm derived tumors comprising many histological subtypes. Most solid childhood cancers belong to these mesoderm derived tumors. They occur at different sites of the body and vary greatly in their aggressive abilities. Three fourths are located in the extremities, most common in thigh, and ten percent each in the trunk wall and peritoneum. The effectiveness of adjuvant chemotherapy remains controversial. Most patients with metastatic sarcoma ultimately progress, thus more specific and less toxic agents are indicated. Detailed histopathological examinations and known clinical prognostic factors have been of limited help in predicting STS outcome. The majority of sarcomas possess complex karyotypes without characteristic genetic changes, but a third of cases possess molecular events implicated in transformation to a malignant...
phenotype. The characterization of defined pathways in sarcomagenesis has fuelled hope for targeted therapies that can significantly alter survival.

Over the last 15 years, we have obtained considerable new knowledge about molecular and biologic features of STS and how these features differ between normal and malignant mesenchymal cells. These differences provide new targets not only for developing novel therapies (e.g. immune cell inhibitors, epidermal growth factor, tyrosine kinase inhibitors, angiogenesis inhibitors), but also for indicating survival prognosis, predicting treatment response, and defining treatment strategies within subgroups of STS and on the individual patient level.

There is increasing evidence of intimate interactions between the neoplastic, malignant tumor cells and cells in the tumor stroma see figure below. In our study we will investigate the profiles of microRNA, both in the malignant mesenchymal cells and cells in the surrounding stoma.

Paracrine interactions between neoplastic cancer cells and supporting cells in tumor stroma (E. Richardsen, 2008).
2. **MicroRNA**

MicroRNA are short (19-25 nucleotides), non-coding, functional RNA molecules that are cleaved from 70 to 100 nucleotide hairpin pre-microRNA precursors in the cytoplasm by RNase III Dicer into their mature form. They regulate gene expression by either repressing translation or cleaving RNA transcripts and can act as tumour suppressors or oncogenes where they are then termed oncomirs. Figure 1 below shows the biogenesis of microRNA, figure 2 shows the microRNA as tumor suppressors or oncogenes (both from Ramiro G et.al. Trends in Molecular Medicine, 2006, 12, 580-7):

An accumulating body of evidence reveals critical functions for microRNAs in various biological processes as diverse as proliferation, apoptosis, and cell differentiation (15) and given their diversity and abundance, microRNAs appear to functionally interact with various components of many cellular networks. Almost 30% of the human genome is estimated to be regulated by microRNAs. Therefore, they are considered as one of the largest classes of gene regulators. We have recently established this method and achieved significant results in a study on the microRNA Has-let-7a in a subgroup of our STS specimens. Several microRNAs are also related to angiogenesis, epithelial-to-mesenchymal transition of
cancer cells and immunosurveillance of the tumor. The miR-17-92 cluster promotes tumor angiogenesis in vivo, while expression of let7-f and microR 27b contributes to in vitro angiogenesis. Relating microRNA results with protein expression will therefore be a novel approach of great interest.

Unique microRNA expression profiles have been able to classify various cancers. In one study, the expression pattern of 217 microRNAs identified cancer type more accurately than messenger RNA. Evidence that microRNA is of critical importance in chemo resistance of several human cancers has just been published. We will investigate related microRNAs in our STS specimens in an attempt to unravel the complex biology of these regulatory mechanisms in correlation to data at hand.

![MicroRNAs as tumor suppressors or oncogenes.](image)

**Figure 2.** MicroRNAs as tumor suppressors or oncogenes. (a) In this model, we propose that a microRNA that normally downregulates an oncogene can function as a tumor suppressor gene when lost in a tumor. The loss of function of this microRNA by mutations, deletions, promoter methylation or any abnormalities in the microRNA biogenesis might result in an abnormal expression of the target oncogene, which subsequently contributes to tumor formation. Some of the proposed mechanisms for inactivation of microRNAs in cancer are experimentally proven, such as the identification of homozygous and heterozygous deletions at 13q14.3 in B-cell CLL, where miR-15a and miR-16-1 are located [9,46]. In addition, germ-line mutations were found in the precursor of this cluster. (b) The amplification or overexpression of a microRNA that downregulates a tumor suppressor or other important genes involved in differentiation might contribute also to tumor formation by stimulating proliferation, angiogenesis and invasion. For example, amplifications of the oncogenic microRNAs, miR-17-92 cluster, miR-21 and miR-155 have been clearly associated with tumor initiation and progression [39,55,56,80].

3. **The aims of the study**

Together with demographic data, clinical data, pathological data and immunohistochemical expression profiles of the tumors we will investigate the predictive and prognostic impact of relevant microRNAs related to immune function, process of epithelial-to-mesenchymal transition of cancer cells (EMT) and angiogenesis.
4. **Patients**

**Study population**
We will use anonymised primary tumour tissue samples from more 800 patients diagnosed or treated with STS during the period 1984 through 2003 at the hospitals in Helse Nord, Norway and the University Hospital of Arkhangelsk, Russia.

**Clinical data**
Demographic, clinical, treatment, and outcome data have been collected from medical records. Patients have at least one year follow-up. The patients had not received chemo- or radiotherapy prior to surgery.

**Inclusion criteria**
- All patients diagnosed or treated with STS at the hospitals in Helse Nord, Norway and the University Hospital of Arkhangelsk, Russia during the period 1984 through 2003.
- Patients with archival untreated tumor tissue samples at Department of Pathology, University Hospital of Northern Norway and Department of Pathology, University Hospital of Arkhangelsk.

**Exclusion criteria**
- Patients without sufficient archival untreated tumor tissue samples of STS at Department of Pathology, University Hospital of Northern Norway and Department of Pathology, University Hospital of Arkhangelsk.

**Outcome**
This study will explore the role of a variety of intracellular molecular markers as predictors of treatment response and indicators of survival prognosis and treatment strategies within subgroups of STS and at the individual patient level by the following endpoints or outcome measurements:
- Response to treatment of subgroups of STS.
- Overall survival of subgroups of STS.
5. Material
Formalin-fixed and paraffin-embedded tumour specimens and control specimens have been obtained from the archives of Departments of pathology, University Hospital of Northern Norway and Department of Pathology, University Hospital of Arkhangelsk.
The stored tumour specimens have been staged according to the International Union Against Cancer (UICC) TNM classification, and histologically subtyped and graded according to the FNCLCC guidelines, as previously described. Prior to inclusion, all tumours and control tissues were reviewed by two independent pathologists.

6. Methods
Tissue Micro array (TMA)
Today, TMAs can be employed for high throughput large-scale investigation of the biologic and prognostic value of molecular marker families. TMA technology allows rapid visualization of molecular targets in hundreds of tumour specimens on a single slide, either at the DNA, RNA, or protein level. The technique facilitates rapid translation of molecular discoveries to clinical applications. TMAs have a number of advantages compared with conventional techniques. The speed of molecular analyses is increased by more than 100-fold, precious tissues are not destroyed and a very large number of molecular targets can be analysed from consecutive TMA sections within a reasonable budget.

Immunohistochemistry (IHC)
In short, IHC staining techniques allow for the visualization of antigens by sequential application of a specific antibody to the antigen. A secondary antibody is linked to the primary antibody to increase the sensitivity of the reaction, and an enzyme complex and a chromogen substrate is added. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site.

Scoring
The degree of protein expression by IHC is graded semiquantitatively and dominant cytoplasmic and membrane staining intensity in both tumor cells and stromal cells are scored as: 0 = negative; 1 = weak; 2 = intermediate; 3 = strong. The cell density of the stroma is scored as: 1 = low density; 2 = intermediate density; 3 = high
density. Slides are examined and scored independently by two trained pathologists blinded to any other pathologic or clinical information. When the data are dissimilar, the cases are reviewed until final agreement is achieved.

Micro RNA analysis
Formalin fixed paraffin embedded tissue samples represent a wealth of potential information on microRNAs in both routine clinical diagnostics and retrospective research analysis. Much of the previous work extracting microRNA has been from fresh patient material, which can be hard to obtain and preserve in adequate numbers. Recently, techniques to make use of formalin fixed and paraffin embedded tissue samples has been developed and their successful application should bring increased speed to the analyses of microRNAs with important diagnostic and prognostic implications.
During the last year we have established the new methods for isolation and assessment of microRNAs from the tumor specimens by using real-time quantitative reverse transcription-polymerase chain reaction (QPCR) analysis. The Recover All Total Nucleic Acid Isolation kit is optimized for the isolation of total nucleic acids (RNA, DNA and micro RNA). This protocol was used to isolate nucleic acids initially from two 1mm sample cores. Since this yielded more than enough RNA the core size was decreased to 0.6mm for both tumour and normal lung tissue.

7. **Statistics and data management**
Sample size, randomisation, and blinding
Sample size was estimated with survival as the primary endpoint. At least a 50% increase in hazard ratio resulting from the presence of a specific marker was assumed to represent a clinically significant effect. The overall 5-year survival for patients with STS is about 70%, and the frequency of a given level of a specific marker is typically about 35%. Analysing the primary endpoint in a proportional hazards regression with a specific marker at a specific level as a dichotomous independent variable, 800 subject are necessary to achieve a power of >80% at an alpha of 5% (PASS 2002, Number Cruncher Statistical Systems, Kaysville, Utah, USA). This estimate does not take into account the testing of multiple markers in the actual analysis, and can only serve as a rough indication of the number of needed subjects. Of 1150 available resected STS patients during the 20-year period
of interest, 800 patients were eligible. When establishing the TMA blocks, original tissue blocks were chosen for sampling in a randomized fashion. The slides have been digitalized into microscopic images (Ariol), and examined and scored independently by two of the authors blinded to any other pathologic or clinical information.

Statistical analysis
Data are presented as mean ± standard error of the mean (SEM). The SPSS for Windows® statistical software package is used to perform the analyses. The IHC scores from each observer were compared for interobserver reliability by use of a two-way random effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient) was obtained from these results. For comparison of more than two groups, variance analysis by one-way ANOVA is used, and patients with missing values for a variable will be excluded from the analysis for that variable. Differences is defined significant when p < 0.05. For correlation analysis, the non-parametric Spearman correlation test is employed. For univariate analysis, the Kaplan and Meier method is used, and statistical significance between survival curves is assessed by the log-rank test. Where appropriate, continuous variables are categorised before analyses, and test for linear trend used for categorical variables with more than two categories. To assess the value of individual pretreatment variables on survival in the presence of all other variables, multivariate analysis will be carried out by using the proportional hazards Cox model.

8. Ethical requirements
Patient data protection and security
During the whole experimental period, each patient is encrypted and given an ID-number. After completion of the trial, all patient information collected in connection to the trial will be stored in the Hospital Central Archive at the University Hospital of Northern Norway. The whole data material will be available for scientific control or later follow up studies.
Ethical committee consent
The National Data Inspection Board, The Regional Committee for Research Ethics, The Cause of Death Registry, and Biobank Board has approved the study.

9. Budget

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10. References


