Metastasis-associated gene signature in primary myxoid liposarcoma identified through a gene expression study

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SUMMARY

Myxoid liposarcoma (MLS) is a rare mesenchymal malignancy with unique extrapulmonary metastatic potential. Although MLS has been associated with specific chromosomal translocations, the factors and pathways regulating metastasis in MLS remain unknown. To identify the molecular mechanisms underlying MLS metastasis, we compared global gene expression profiles of primary tumor tissues from MLS patients with different metastatic statuses using DNA microarray analysis. In total, 393 genes were differentially expressed between the tumors from four patients with metastasis and those from 11 patients without metastasis. Differentially expressed genes were functionally annotated based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Supervised classification based on the 393 genes clearly discriminated samples according to metastatic status. The pathways responsible for metastasis included “focal adhesion,” “pathways in cancer,” “ECM-receptor interaction,” and “tight junction.” The differential expression of alpha-synuclein was confirmed at the protein level; the protein was downregulated in metastatic MLS. Meta-analysis revealed that MLS could be discriminated from the other sarcomas based on the expression of the metastasis-associated genes. The metastasis-associated genes identified in this study are worthwhile further investigation to further our understanding of MLS and are expected to lead to novel clinical applications for MLS.

Key words: myxoid liposarcoma, metastasis, microarray, gene expression signature, alpha-synuclein

INTRODUCTION

Myxoid liposarcoma (MLS) is one of the most common sarcomas; it accounts for one-third of all liposarcomas and 10% of all adult soft-tissue sarcomas9. Clinical features of MLS are diverse, ranging from localized, curable tumors to metastatic ones, which can cause death9. Because of this clinical diversity, patient stratification is critical to optimizing treatment. Previous studies have revealed important pathological and molecular signatures of MLS, which were associated with clinical outcome. For example, a significant association of the presence of rounded cells with poor prognosis in MLS has been reported9. Soft-tissue sarcomas tend to metastasize to the lung, and metastases to other sites are typically observed in the advanced stages. However, MLS shows unique metastatic patterns to bones such as the spine and other soft tissues such as the retroperitoneum, limbs, and axilla4–7. Unique molecular characters in MLS, such as the recurrent chromosomal translocation t(12;16)(q13;p11), and chimeric proteins, such as the FUS-CHOP fusion, have been considered as trabectedin targets8, 9). Besides these clinical, pathological, and molecular observations, the underlying mechanisms of metastasis and poor prognosis in MLS remain largely obscure, and further investigation of the molecular aberrations in MLS is required to achieve better clinical outcomes.

To identify genes responsible for metastasis in MLS, we performed a global mRNA expression analysis through mi-
croarray experiments. We compared the primary tumor tissues from MLS patients with different metastasis statuses to detect differentially expressed genes.

MATERIALS AND METHODS

Patients

This study included 15 patients with MLS who underwent curative resection at the National Cancer Center Hospital of Japan. The patients did not receive adjuvant chemotherapy. The patients’ clinical characters are summarized in Table 1. This study was approved by the ethical committee of the National Cancer Center, and informed consent was obtained from all patients participating in this study.

Gene expression profiling

Total RNA was extracted from frozen tumor tissues using the RNeasy kit (Qiagen, Venlo, the Netherlands). In brief, frozen tissues were crushed and powdered in liquid nitrogen using a Multi-beads Shocker (Yasui-kikai; Osaka, Japan). mRNA expression profiles of the samples were obtained by hybridizing the RNA to the SurePrint G3 Human GE DNA microarray (8×60K, Ver3.0, Agilent Technologies, Santa Clara, CA), following the manufacturer’s instructions. Hybridized microarrays were scanned with a microarray scanner (Agilent G2565BA) with default protocols and settings.

Statistical analysis of microarray data

The microarray data were normalized and standardized using the Bioconductor limma package (http://bioconductor.org/packages/limma/). Differences between two sample groups were established using an unpaired t-test. A p-value<0.05 was considered significant.

Western blotting

Protein expression levels were examined by western blotting. In brief, frozen tissues were crushed and powdered in liquid nitrogen using the Multi-beads Shocker, after which proteins were extracted with urea lysis buffer (6 M urea, 2 M thiourea, 3% CHAPS, and 1% Triton X-100). After centrifugation at 15,000 rpm for 30 min, the supernatant was recovered as total protein extract. The proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; ATTO, Tokyo, Japan), transferred to a PVDF membrane, and reacted with specific antibody against alpha-synuclein (SNCA) (610786, BD Transduction Laboratories) overnight. Then, the blots were incubated with secondary antibody conjugate with horse radish peroxidase, and the immune complex was detected by chemiluminescence using the ECL Prime kit (GE Healthcare Sciences). The image was captured with an Image Analyzer (GE Healthcare Sciences).

Meta-analysis

mRNA expression data were obtained from the Gene Expression Omnibus data file GSE30929. The expression levels of specific genes were used to group liposarcomas with different histological appearance. Those included well-differentiated liposarcoma, dedifferentiated liposarcoma, pleomorphic liposarcoma, and myxoid liposarcoma. Hierarchical clustering and principle component analysis (PCA) were done using the data-mining software Expressionist (Genedata AG, Basel, Switzerland).

Data analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations were inferred using the Database for Annotation, Visualization and Integration Discovery (DAVID) software (http://david.abcc.ncifcrf.gov/)[10]. KEGG analysis results were plotted using the R package “tree-map”[11].

| Table 1. The patients’ clinical and pathological characters |
|-----------------|--------|---------|-----------------|-----------------|-----------------|-----------------|-----------------|
| sample number  | age    | gender  | primary tumor site | metastatic status | site of metastasis | metastasis free period (month) | oncological outcomes |
| 1              | 65     | male    | foot             | negative          | none             | 10              | DOD*             |
| 2              | 58     | male    | thigh            | negative          | none             | 36              | CDF              |
| 3              | 43     | female  | lower leg        | negative          | none             | 159             | CDF              |
| 4              | 35     | female  | lower leg        | negative          | none             | 88              | CDF              |
| 5              | 36     | male    | buttok           | negative          | none             | 32              | AWD              |
| 6              | 53     | male    | thigh            | negative          | none             | 17              | CDF              |
| 7              | 57     | male    | thigh            | negative          | none             | 63              | CDF              |
| 8              | 38     | male    | thigh            | negative          | none             | 14              | CDF              |
| 9              | 39     | female  | thigh            | negative          | none             | 0               | CDF              |
| 10             | 65     | male    | thigh            | negative          | none             | 77              | CDF              |
| 11             | 47     | female  | thigh            | negative          | none             | 68              | CDF              |
| 12             | 61     | female  | thigh            | positive          | abdomen          | 17              | AWD              |
| 13             | 31     | male    | forearm          | positive          | multiple bones   | 65              | DOD              |
| 14             | 42     | male    | lower leg        | positive          | vertebra         | 1               | NED              |
| 15             | 69     | male    | buttok           | positive          | chest wall       | 7               | DOD              |

DOD: dead of disease, AWD: alive with disease, CDF: continuous disease free, NED: no evidence of disease
* Died of bladder carcinoma
RESULTS

Identification of differentially expressed genes

To explore the molecular background of metastasis in MLS, we examined the global mRNA expression using DNA microarrays. In total, 393 genes showed different expression levels between the 4 primary tumors with metastasis and the 11 primary tumor tissues without metastasis; 189 and 204 genes were significantly up- and downregulated in the tumors with metastasis by more than 1.5-fold. Fig. 1 shows a waterfall plot of the gene expression differences. The raw microarray data and a list of the 393 genes are presented in Supplementary Tables 1. To recognize and visualize transcriptomic patterns on the basis of these 393 genes, we carried out supervised clustering (Fig. 2). Hierarchical clustering revealed that all 4 tumors with metastasis and 9 out of 11 tumor tissues without metastasis clustered together according to metastasis status (Fig. 2A). PCA distinguished all samples according to their metastatic status based on the expression pattern of the 393 genes (Fig. 2B). We concluded that these genes may represent overall molecular features of tumor tissues with regard to metastatic status.

We functionally classified the 393 genes based on KEGG pathway analysis and mapped them onto KEGG pathways. Fig. 3 visualizes the results as an area-based treemap, which structures hierarchical data as a set of nested rectangles. The size of a rectangle is proportional to the number of genes assigned to the correlating pathway, and the panel color represents the degree of relevance. The dominant pathways were “focal adhesion,” “pathways in cancer,” “ECM-receptor interaction,” and “tight junction.” The assignments of individual genes to the pathways are summarized in Supplementary Table 2.

SNCA protein expression in MLS

We selected SNCA from the 393 genes for validation of the expression difference by western blotting. Overexpression of SNCA has been previously associated with metastasis in melanoma12, 13). In contrast, in our study, SNCA was downreg-

![Fig. 2. Clustering analysis of the tumor samples with different metastatic status.](image)

The tumor samples are grouped on the basis of mRNA expression of the 393 genes by hierarchical clustering (A) and PCA (B). In hierarchical clustering, 9 out of 11 tumors without metastasis and the 4 tumors with metastasis are grouped according to their metastasis status (A). All tumors are classified according to the metastasis status in PCA (B).
Similarly, PCA discriminated MLS from the other liposarcoma samples such as dedifferentiated liposarcoma, pleomorphic sarcoma, and well-differentiated liposarcoma (Fig. 5B). These observations suggested that the genes associated with metastasis in MLS may represent a transcriptomic background unique to MLS.

**DISCUSSION**

This study identified genes associated with the metastatic features of MLS, reflecting its unique extrapulmonary metastatic potential. The identification of the genes responsible for this metastatic behavior is a first step in furthering our understanding of the molecular background of MLS.

To our knowledge, this is the first gene expression study to focus on metastasis-associated genes in MLS. Furthermore, by performing a meta-analysis of the genes identified in this study, we demonstrated the unique expression features of these genes, which allowed the discrimination of MLS from other liposarcomas. The raw microarray data are added as a supplement to this paper; we believe that they will be a useful resource for MLS studies for integration with other gene expression data.
In this study, for the first time, we observed the downregulation of SNCA in metastatic MLS, indicating that the function of SNCA may depend on the malignancy type.

The highlight of this study was that the MLS samples were effectively discriminated from the other liposarcomas considered based on the expression patterns of genes associated with metastasis in MLS. Although soft-tissue sarcomas tend to metastasize to the lungs, MLS shows unique metastatic patterns to the spine and soft tissues other than the lungs. The genes identified in this study will be worth investigation and might offer alternative to the current treatment strategy.

Our study had several limitations that should be acknowledged. First of all, it lacked sufficient statistical power to establish the relationships of gene expression with clinical and pathological characteristics of MLS owing to the small sample set. In addition, for the same reason, a stratification of samples according to the metastasis site was not feasible. A larger sample size is required to obtain conclusive results; however, because MLS is quite rare, it is difficult to acquire a sufficiently large number of MLS samples in a single institutional study; meta-analysis might offer a solution in this regard. Secondly, this study involved a retrospective analysis, which may lead to subject selection bias. Prospective studies are preferable, and offer higher reliability of the results. Thirdly, although the expression study suggested functional contributions of the identified 393 genes to metastasis in MLS, we did not verify any functional roles in metastasis in vitro, because no in vitro model of MLS is publicly available at present. We extensively searched for MLS cell lines in public cell banks; however, we could not find MLS lines in the American Type Culture Collection (ATCC, http://www.

The genes associated with metastasis in this study were classified into several pathways or fundamental cellular functions; mainly, “focal adhesion,” “pathways in cancer,” “ECM-receptor interaction,” and “tight junction.” Various lines of evidence have suggested the implication of focal adhesion as well as extracellular matrix proteins in the metastasis process, and these have been investigated as therapeutic targets. The genes for focal adhesion included IGF1R and PDGFRA, and the cancer pathway proteins included FGFR2, IGF1R, and PDGFRA (Supplementary Table 2). Drugs targeting these proteins have already been clinically implemented for the treatment of various types of sarcomas. The possible application of anti-FGFR2, -IGF1R, and -PDGFRA drugs to MLS should be further explored in preclinical studies.

We confirmed the down-regulation of SNCA in metastatic MLS. SNCA is a small protein that localizes to synaptic terminals, where it associates with microtubules. SNCA is unique in its self-oligomerization and self-aggregation, which are attributable to posttranslational modifications such as phosphorylation, oxidation, and sumoylation. It is noteworthy that SNCA seems to play an important role in the progression of neurodegenerative diseases such as Parkinson’s disease and hereditary amyloidosis. Moreover, SNCA was the protein the most significantly upregulated by radiation therapy in prostate cancer, suggesting its utility as a biomarker. The aggregation of SNCA has been attributed to ER stress and oxidative stress, which consistently exist in cancer cells. Epidemiological studies have suggested a link between melanoma and Parkinson’s disease. Further, Matsuo et al. identified a specific SNCA expression pattern in melanoma cell lines. Using primary tumor tissue samples, Welinder et al. confirmed the association of SNCA with metastasis in melanoma. In this study, for the first time, we observed the downregulation of SNCA in metastatic MLS, indicating that the function of SNCA may depend on the malignancy type.

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atcc.org), The European Collection of Cell Cultures (ECACC, http://www.phe-culturecollections.org.uk), Interlab Cell Line Collection (ICLC, http://www.iclc.it), German Collection of Microorganisms and Cell Cultures (DSMZ, http://www.dsmz.de), Cell Resource Center for Biomedical Research (IDAC, http://www2.idac.tohoku.ac.jp), Japan Collection of Research Bioresources (JCRB, http://cellbank.nibio.go.jp), and Riken Bioresource Center (BRC, http://www.brc.riken.jp)31). Patient-derived cancer models of MLS will have to be established to functionally verify the results of our expression study.

CONCLUSIONS

We demonstrated the presence of a gene expression signature for metastasis in the primary tumor tissues of MLS. The identified metastasis-associated genes may represent molecular features unique to MLS, and allowed discrimination from the other liposarcomas. Our data and results will be a useful resource for future studies furthering our understanding of mechanisms underlying MLS.

DECLARATIONS

Ethics approval and consent to participate

This study was approved by the ethical committee of the National Cancer Center, and informed consent was obtained from all patients participating in this study.

Consent for publication

The patients participating in this study agree to use their clinical materials and publish the results of study using their clinical materials.

Availability of data and material

All data generated, except DNA microarray data, during this study are included in this published article and its supplementary information files. DNA microarray data are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Ethics, consent and permissions and Consent to publish

This study was approved by the ethical committee of the National Cancer Center, and informed consent was obtained from all patients participating in this study. This study does not include the individual patient data. The participants agreed to publish the results of research where their clinical materials are used.

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ABBREVIATIONS

MLS: myxoid liposarcoma, SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SNCA: synuclein, PCA: principle component analysis, KEGG: Kyoto Encyclopedia of Genes and Genomes, DAVID: Database for Annotation, Visualization and Integration Discovery, ER: endoplasmic reticulum, ATCC: American Type Culture Collection, ECACC: European Collection of Cell Cultures, ICLC: Interlab Cell Line Collection, GSMZ: German Collection of Microorganisms and Cell Cultures, IDAC: Cell Resource Center for Biomedical Research, JCRB: Japan Collection of Research Bioresources, BRC: Riken Bioresource Center

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