Down-regulation of Plakoglobin in Soft Tissue Sarcoma is Associated with a Higher Risk of Pulmonary Metastasis

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Abstract. Soft tissue sarcomas (STS) behave with aggressiveness and metastatic potential, that can vary depending on their locations. There has been little information on the exact molecular mechanisms involved in their biological aggressiveness. To identify genes involved in the differences, the gene expression profiles were compared between STS-orthotopic and heterotopic implanted models, and their significance in human STS was verified. Human fibrosarcoma HT1080 cells were implanted either in the quadriceps femoris muscles or footpads of nude mice, and the gene expression profiles of the tumors were compared by cDNA arrays. The mRNA and protein levels of the identified genes were examined by both real time RT-PCR and immunohistochemistry not only in the tumors of the models, but also in clinical STS. The implanted HT1080 cells demonstrated different growth and metastatic potentials depending on their implant locations. cDNA array analyses showed decreased expression of the plakoglobin gene in the intramuscle-implanted group, which was statistically confirmed by real-time RT-PCR (p=0.04). Plakoglobin was immunolocalized diffusely in the cytoplasm of tumor cells implanted in the footpads, but not those in the muscle. Real-time RT-PCR assays of clinical STS showed that the mean plakoglobin/glyceraldehyde 3-phosphate dehydrogenase (G3PDH) ratio in primary sarcoma tissues with pulmonary metastases (0.92) was significantly lower than in those without metastasis (6.58) (p<0.0001), and that STS cases with high plakoglobin gene expression had an excellent prognosis. These results suggest that plakoglobin gene expression level might be useful as a new biomarker for metastasis and prognosis of human STS.

Soft tissue sarcomas (STS) frequently metastasize to the lungs, and pulmonary metastasis is one of the most critical factors that determine the prognosis of patients with STS. However, knowledge of the mechanisms of STS metastasis is far from satisfactory because of the relatively low incidence and high heterogeneity of STS (1, 2).

Tumor metastasis is a complicated phenomenon composed of several steps (3). In order to investigate the molecular mechanisms involved in each step, various kinds of experimental animal models have been developed (4). The use of orthotopic implantation models has recently become popular for research on common carcinomas (4, 5). Fidler et al. implanted human renal carcinoma cells either subcutaneously or into the subcapsular portion of the kidneys of nude mice and found that the rate of pulmonary metastases was increased in the latter (6). An increased metastatic rate to clinically relevant organs was reported in an orthotopic spontaneous metastatic model for other carcinoma cell lines (4, 7). They are still rarely used for sarcoma research, although Khanna et al. established an orthotopic implanted model for osteosarcoma and investigated the expression of metastasis-related genes (8). It has been postulated that the microenvironment may influence the regulation of gene expression involved in invasion and metastasis as well as the proliferation and apoptosis of tumor cells. In STS, a deep location has been shown to be one of the significant prognostic factors by previous clinicopathological studies (9). However, little information on the genes that play pivotal roles in the metastatic abilities of STS cells depending upon the depth of their location is available.
Plakoglobin (gamma-catenin) is a major component of the submembranal plaque of desmosomes and adherens junction in mammalian cells (10). Plakoglobin, like its close homologue beta-catenin, interacts with cadherins to mediate cell-cell adhesion. It is also associated with a transcription factor of the T-cell factor/lymphocyte enhancer factor (TCF/LEF) family and regulates the expression of target genes involved in proliferation and cell fate determination (11, 12). While elevated beta-catenin expression has been implicated in hyperproliferation and tumor formation, overexpression of plakoglobin has been shown to suppress cell proliferation and tumorigenicity in experimental animals (13). The reduced expression of plakoglobin observed in primary tumor tissues and metastatic lesions of esophageal, nasopharyngeal, urinary, pulmonary, thyroid and skin carcinomas, and the loss of heterozygocity in breast and ovarian carcinomas indicate that it is a tumor suppressor (14-21). The down-regulation of plakoglobin was reported to be a marker for both tumor growth and metastasis in oral and pharyngeal squamous cell carcinomas and non-small lung cancer (17, 18).

In this study, differential gene expression profiles between STS-orthotopic and heterotopic implanted models using the human fibrosarcoma HT1080 cell line were investigated and their significance in human STS was verified.

**Materials and Methods**

**Cell culture and tumor growth in BALB/c nude mice (STS-orthotopic and heterotopic spontaneous metastasis models).** The human fibrosarcoma HT1080 cell line (American Type Culture Collection, Rockville, MD, USA) was maintained as a subconfluent monolayer culture in RPMI 1640 medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% fetal calf serum at 37°C under 5% CO₂ / 95% air. The medium was exchanged every third day, and passing was routinely performed when the culture reached 80-85% confluency. The HT1080 cells were harvested by trypsinization. Thirteen four- to six-week-old, female athymic BALB/c nude mice (purchased from Clea Japan Inc., Tokyo, Japan) were injected with 5x10⁵ cells in a total volume of 50 µl RPMI 1640 medium either into the quadriceps femoris muscle (n=4) or the footpads (n=9). The lungs were harvested 6 weeks later and subjected to cryostat sectioning. The number of metastatic nodules was counted in three representative, maximal coronal sections with 200 µm distance between slices and stained with Haematoxylin and Eosin (HE). Nodules formed of at least five tumor cells were defined as a micrometastasis.

**RNA extraction, cDNA arrays, probes, hybridization, and scanning.** Tumor samples in the muscle (n=2) and the footpad (n=2) were harvested 6 weeks after implantation for RNA extraction. Tumor tissues not in the center, but at the periphery including some surrounding reactive non-tumor area were dissected to investigate gene expression in the tumor-stromal microenvironment. The total RNA was isolated using the Atlas Pure Total RNA labeling system (BD Biosciences Clontech, Japan). To make isotope-labeled cDNA probes by reverse transcription, the total RNA from each sample was incubated in a cocktail containing deoxyadenosine triphosphate (dNTP) mix (for deoxyadenosine triphosphate (dATP) label), cDNA synthesis (CDS) primer mix (Atlas array-specific primers, BD Biosciences Clontech) and [α-32P] dATP (Amersham Pharmacia Biotech Japan, Tokyo, Japan). The 32P-labeled probes were purified using a BD Atlas Nucleospin extraction kit (BD Biosciences Clontech, Japan), and the radioactivities on the membranes were measured using an imaging analyzer (BAS-5000, Fujifilm Co., Tokyo, Japan). Differentially expressed genes from the two samples were compared grossly and analyzed by the Array Gauge Ver.1.21 (Fujifilm Co.). The expression levels from the two were compared at each spot and the differences analyzed in *siliro*. The experiment was repeated twice to ensure reproducibility.

**Quantitation of plakoglobin gene mRNA expression.** The total RNA was extracted using the same method as described above and the quality of the total RNA was assessed using a RNA 6000 Nano Labchip kit on a Bioanalyzer 2100 (Agilent Technologies Japan, Tokyo, Japan). Specific primers and probes for plakoglobin (Hs00158408 m1) and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Hs99999905 m1), pre-designed by Assay-On-Demand Gene Expression Products, were purchased from Applied Biosystems (Applied Biosystem Japan, Tokyo, Japan). About 0.5 µg of total RNA from each sample was used to perform the reverse transcription reaction. TaqMan Reverse Transcription Reagents (Applied Biosystem Japan) were used according to the manufacturer's instructions (25°C 10 min, 48°C 30 min, 95°C 5 min). The reverse transcription product (1 µl) was used to perform real-time quantitative PCR in a reaction volume of 50 µl (TaqMan PCR core reagent kit, Applied Biosystem Japan) using the ABI PRISM 7700 Sequence Detection System (Applied Biosystem Japan). The PCR protocol was according to the manufacturer's recommendations (50°C 2 min, 95°C 10 min, 95°C 15 sec, 60°C 1 min), 40 cycles). The expression levels of plakoglobin mRNA were determined by the relative standard curve method according to the directions of the manufacturer. Briefly, two standard curves for both plakoglobin and G3PDH were obtained from the amplification of total RNA of the tumors arising from the HT1080 cell line in the footpad. Based on each amplification plot for plakoglobin and G3PDH, a threshold cycle (TC) value was calculated representing the PCR cycle number at which the fluorescence was detectable above an arbitrary threshold. The relative amounts of plakoglobin mRNA were quantified by linear extrapolation of the TC values using the equation of the line obtained from the plakoglobin standard curve. These values were then divided by the relative amounts of G3PDH. These normalized amounts of plakoglobin (the relative amount of plakoglobin mRNA / the relative amount of G3PDH mRNA) were defined as the expression levels of plakoglobin mRNA.

**Immunohistochemical analysis.** Plakoglobin protein expression in the tumor tissues either in the muscle or footpad was examined by immunohistochemistry using the streptavidin-biotin-peroxidase-complex method (Histofine SAB-PO kit, Nichirei Co., Tokyo,
Tumor specimens and patients. For real-time RT-PCR, macrodissected specimens were obtained from 33 patients (30 from the primary lesion and 3 from metastatic pulmonary nodules) with malignant STS who underwent surgery at the Kanazawa University Hospital between 1997 and 2004 (Table I). The Institutional Review Board at Kanazawa University approved this study. All the patients provided informed consent. The diagnoses were confirmed as defined by the World Health Organization criteria. The specimens came from 19 men and 14 women with a mean age of 53 years (range, 12-87 yrs). There were 17 cases of malignant STS whose histological diagnosis was malignant fibrous histiocytoma (MFH), 6 cases of liposarcoma, and 10 cases of various other types. Fifteen (2 M1 cases) of the 30 primary tumor patients developed pulmonary metastases. Fourteen of the 30 patients received some chemotherapy before surgery, and one patient received it only after surgery. Four samples from the group of 14 patients who received neo-adjuvant chemotherapy were taken from their biopsy which was performed prior to the administration of chemotherapy. The remaining 10 samples were obtained at the time of surgery after the administration of chemotherapy (4 responders and 6 non-responders). As for the effects of preoperative chemotherapy, an objective response was defined as >50% tumor shrinkage radiographically or a histological response (grade III or grade IV) (22, 23). The median postoperative follow-up time was 34 months (range, 2-81 months). The pathological stages were classified according to the World Health Organization histological classification system for STS and were staged according to the Musculoskeletal Tumor Society Staging System.

Statistical analysis. The clinical end-points examined were the time from treatment initiation to the time of pulmonary metastasis (metastasis-free survival). The data are presented as the mean±SEM. For comparisons between the two groups, the Mann-Whitney U-test was used to statistically analyze the results. The survival rates were calculated using the Kaplan-Meier method and compared with the log-rank test. A Cox proportional hazards model was used for univariate and multivariate survival analysis to assess the independent prognostic significance of the clinicopathological variables, including plakoglobin mRNA expression level. The cut-offs for low and high level of expression were pre-established according to the median value of these markers in a preliminary series of all tumors (24). A p-value of <0.05 was considered statistically significant. All the statistical analyses were performed using Statview software (SAS Institute Inc., Cary, NC, USA; http://www.sas.com).

Results

Tumor proliferation and pulmonary metastasis by HT1080 cells implanted into the quadriceps femoris muscles or footpads of nude mice. The tumorgrowth in the muscle was much larger than that in the footpad (data not shown). Histologically, the tumors in the muscle were comprised of short spindle-shaped tumor cells and showed central necrosis and invasive borders, while those in the footpad were composed of plump shaped tumor cells and showed expansive borders. The tumors implanted in the muscle exhibited a much higher incidence of pulmonary metastases than those in the footpad (21.0±7.2 and 3.3±1.2, respectively; p=0.0133; Mann-Whitney U-test) (Figure 1).

DNA array. In general, DNA array analysis showed close similarity in gene expression patterns and there was little variation between the tumors implanted in the muscle and those in the footpad, as expected, because the same cell line was implanted in the two sites. Compared to the neighboring spots, however, several genes exhibited differences in each analysis. Of these, only plakoglobin gene expression was constant in two analyses. The expression of the plakoglobin gene was decreased in the tumors implanted into the muscles compared to those in the footpad (21.0±7.2 and 3.3±1.2, respectively; p=0.0133; Mann-Whitney U-test) (Figure 2). In the silico analysis of the two arrays, the mean muscle/footpad density ratio of the plakoglobin gene were 0.5419 and 0.3256, respectively.

Confirmation of expression levels of plakoglobin gene by real-time RT-PCR. To confirm the DNA array data, the expression of the plakoglobin gene in both tumor tissues was quantitatively assayed by real time RT-PCR. The plakoglobin/G3PDH ratio in the muscle group was significantly lower than that in the footpad group (0.18±0.00 x 10^3, 1.14±0.33 x 10^3, respectively; p=0.04, Mann-Whitney U-test).

Immunohistochemical expression of plakoglobin in tumor cells implanted in the footpad and the muscle. The HT1080 cells implanted in the footpad of the BALB/c nude mice exhibited an intense positive reaction in the cytoplasm diffusely for anti-gamma-catenin (plakoglobin), while the reaction was much weaker in the tumor cells implanted in the quadriceps muscle (Figure 3). A positive reaction was also detected in the epidermal cells and some endothelial cells.
Association of plakoglobin mRNA expression level with metastases in highly malignant STS cases. The results of quantitative RT-PCR of mRNA expression of the plakoglobin gene in 30 primary malignant STS cases and three pulmonary nodules are shown in Table I. The plakoglobin expression in all the primary tumors ranged from 0.04 to 18.98 (median 2.19, mean 3.75), while the mean plakoglobin expression levels in the primary lesions which did not develop pulmonary metastases, the primary lesions which did develop pulmonary metastases and in the metastatic pulmonary nodules, were 6.58 (SD 5.42, SE 1.40), 0.92 (SD 1.07, SE 0.28) and 1.41 (SD 0.69, SE 0.40), respectively (Figure 4a). The primary lesions that developed pulmonary metastases and the pulmonary metastatic nodules had lower plakoglobin gene expression. The plakoglobin gene mRNA levels were significantly correlated with the clinical stage of the STS (p<0.0001; using the Mann-Whitney U-test). The expression of plakoglobin mRNA did not show any correlation with tumor size of the
primary tumors (data not shown). With regard to histological subtypes, the expression levels of the plakoglobin gene in the synovial sarcoma patients with metastases tended to be slightly higher than the other cases of sarcoma with metastases.

The cut-off between low and high levels of expression in the Kaplan-Meier analysis was set at 2.2 based upon the median value, 2.19. The Kaplan-Meier metastasis-free survival curves for gene expression dichotomized by the median level for plakoglobin gene are shown in Figure 4b. The metastasis-free 1-year and 2-year survival rates of the patients in the low plakoglobin group were 53% and 27%, respectively. On the other hand, both were 87% in the patients in the high plakoglobin group. There was a significant association between the development of pulmonary metastases and the expression levels of plakoglobin gene ($p=0.001$; log-lank test). The results of univariate and multivariate analysis for metastasis-free survival according to various clinicopathological factors, including plakoglobin gene expression levels, are summarized in Table II. Only a high level of plakoglobin gene expression was a statistically significant favorable prognostic factor in this series (relative risk in multivariate analysis $0.50$, 95% CI $0.29-0.84$; $p=0.010$).

Figure 4c shows that the patients with high plakoglobin mRNA expression levels had a much better disease-specific survival compared with the patients with low levels. A $p$-value is not available because no patient in the high plakoglobin group has yet died. The disease-specific 2-year and 5-year survival rates of the patients in the low plakoglobin group were 66% and 55%, respectively. On the other hand, both were 100% in the patients in the high plakoglobin group. Thirteen (87%) of the 15 patients in the low plakoglobin group have developed pulmonary metastases, while only 2 (13%) of the 15 in the high plakoglobin group have done so.

**Discussion**

There has been no previous report on an orthotopic implantation spontaneous metastasis model for STS. The present comparative study between the orthotopic and heterotopic implantation spontaneous models clearly showed that the HT1080 STS cell line behaved differently not only in tumor growth characteristics, but also in invasiveness and metastatic potential to the lungs. The results confirmed the applicability of this model to the investigation of genes involved in the pulmonary metastases of STS. The cDNA array analysis selected plakoglobin as a tumor suppression gene for STS cells. Furthermore, the mRNA expression levels of the plakoglobin gene in the clinical STS samples correlated with the development of pulmonary metastasis, but not tumor size, again suggesting that plakoglobin is a metastasis suppression gene, and that its expression levels may provide prognostic information on the metastatic potential of human STS. Amitay et al. examined immunohistochemically the expression of plakoglobin in neuroblastoma cell lines and tumors and demonstrated that the loss of plakoglobin expression was correlated with an adverse prognosis (25). Gastaldi T et al. showed differential expression of plakoglobin in rhabdomyosarcoma (RMS) cell lines and tumor samples.
Figure 2. Autoradiography of hybridization results of cDNA reverse transcribed from the tumor of HT1080 in the footpad (a) and that in the muscle (b). Arrows indicate differential gene expression of only one gene (plakoglobin).
Figure 3. Photomicrographs showing immunohistochemical staining of plakoglobin (gamma-catenin) in the tumor of the footpad (a) and the muscle (b). The tumor tissue in the muscle had lower plakoglobin protein expression in the cytoplasm than tumor tissue in the footpad. The plakoglobin protein was localized in the cytoplasm of tumor cells. Scale bar=50 µm.
Plakoglobin was expressed in embryonal RMS cell lines and tumors, whereas it was absent and/or detectable at an extremely low level in alveolar RMS which is more aggressive and has a higher tendency to metastasize. The presented study included a case of alveolar RMS (Table I, Case 29) in which the plakoglobin/G3PDH mRNA ratio in this study was 2.4, which was low but still more than 2.2. The patient was alive without pulmonary metastases at follow-up 59 months after surgery. Our data are consistent with the previous data and provide additional evidence that plakoglobin plays a metastasis suppressing role in highly malignant STS, and may provide a novel prognostic parameter in STS. A clinicopathological study of plakoglobin on a large number of human STS will be needed to establish the prognostic value of plakoglobin mRNA expression.

The expression of plakoglobin in the HT1080 cell line implanted in the footpad was not localized in the cellular junctions, as observed in carcinoma cells, but diffusely distributed in the cytoplasm. This was consistent with the data in embryonal RMS (26). Simcha and colleagues reported that the transfection of the plakoglobin gene into a human renal cell carcinoma cell line which did not express cadherins, plakoglobin, alpha-catenin or beta-catenin resulted in the suppression of tumor formation in nude mice (13). In these cells, plakoglobin did not exhibit junctional localization, but was diffusely distributed in the cytoplasm and nucleus, possibly indicating that the anti-tumorigenic activity of plakoglobin in these carcinoma cells might be associated with its signaling activity, rather than with its function in cellular adhesion. The cytoplasmic expression of plakoglobin in the HT1080 cell line and STS tumors also seemed to be associated with the signaling activity of pivotal metastasis-related genes. The present immunostaining for plakoglobin did not detect plakoglobin in the nuclei the HT1080 cells. It is possible that relatively small amounts of nuclear plakoglobin are sufficient for its signaling activity.

Gastaldi et al. observed that the differential expression of plakoglobin between alveolar and embryonal rhabdomyosarcoma was regulated by DNA methylation and histone acetylation (26). Histone deacetylase (HDAC) plays a key role in gene expression by suppressing the transcription of a number of target genes. Shim et al. identified, in the HT1080 cell line, the plakoglobin gene as a new target of HDAC using a cancer-gene focused gene microarray. Induction of plakoglobin by the inhibition of HDAC activates the Wnt signaling pathway through T-cell factor/lymphocyte enhancer factor (TCF/LEF) reporter gene expression (27). These data indicate that the plakoglobin level is regulated by DNA histone acetylation status, suggesting that HDAC may be one of the critical target molecules for the down-regulation of the transcription of metastasis-related genes in human STS (28). Cytoplasmic plakoglobin also seems to be a signaling activator and recently, Li et al. demonstrated that the intracytoplasmic plakoglobin gene modulated the induction of bcl-2 in a SCC cell line (29).
Our results from the clinical samples showed that the incidence of lung metastases were significantly higher in the group of patients with low plakoglobin mRNA expression compared with the patients with high expression and that the survival of patients with lower plakoglobin expression was lower than those with higher expression. The data showing that three lung nodules also had low expression of plakoglobin (Table I), indicated that down-regulation of plakoglobin expression might provide favorable conditions for the development of metastases. Clinically, even with the same histological malignancy, some patients with STS develop metastases months or years after the resection of the primary lesion and succumb to metastatic disease. Our results might be used to guide the treatment of patients with STS; for a patient without lung metastases, low plakoglobin expression would suggest a high risk for their subsequent development, so they might be candidates for more aggressive treatment. Furthermore, plakoglobin might be a good biomarker because plakoglobin is involved in an early step of metastasis formation and positively correlated with metastatic potential. However, this study was limited by an insufficient number of samples and follow-up period for the patients. Little is known about the metastasis-related genes modulated by plakoglobin, both in vitro and in vivo studies will be needed to provide such information.

In conclusion, our results suggest that down-regulation of plakoglobin mRNA expression is related to a higher risk of lung metastasis. The plakoglobin gene expression level in STS was identified as a new biomarker for metastasis.

Acknowledgements

We thank Dr. Gong Mign (an overseas student from China-Japan Friendship Hospital to Kanazawa Medical University) for her technical support. This work was supported in part by the Japanese Ministry of Education, Culture, Sports, Science and Technology (to YU), and a grand from Kanazawa Medical University (to YU, HTRC Grant H2007-10).

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Received November 5, 2007
Revised December 31, 2007
Accepted January 9, 2008