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Clear cell carcinoma of the ovary: Is there a role of histology-specific treatment?

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Abstract

Several clinical trials to establish standard treatment modality for ovarian cancers included a high abundance of patients with serous histologic tumors, which were quite sensitive to platinum-based chemotherapy. On the other hand, ovarian tumor with rare histologic subtypes such as clear cell or mucinous tumors have been recognized to show chemo-resistant phenotype, leading to poorer prognosis. Especially, clear cell carcinoma of the ovary (CCC) is a distinctive tumor, deriving from endometriosis or clear cell adenofibroma, and response rate to platinum-based therapy is extremely low. It was implied that complete surgical staging enabled us to distinguish a high risk group of recurrence in CCC patients whose disease was confined to the ovary (pT1M0); however, complete surgical staging procedures could not lead to improved survival. Moreover, the status of peritoneal cytology was recognized as an independent prognostic factor in early-staged CCC patients, even after complete surgical staging. In advanced cases with CCC, the patients with no residual tumor had significantly better survival than those with the tumor less than 1 cm or those with tumor diameter more than 1 cm. Therefore, the importance of achieving no macroscopic residual disease at primary surgery is so important compared with other histologic subtypes. On the other hand, many studies have shown that conventional platinum-based chemotherapy regimens yielded a poorer prognosis in patients with CCC than in patients with serous subtypes. The response rate by paclitaxel plus carboplatin (TC) was slightly higher, ranging from 22% to 56%, which was not satisfactory enough. Another regimen for CCC tumors is now being explored: irinotecan plus cisplatin, and molecular targeting agents. In this review article, we discuss the surgical issues for early-staged and advanced CCC including possibility of fertility-sparing surgery, and the chemotherapy for CCC disease.

Keywords: Review, Ovarian cancer, Clear cell carcinoma, Surgical staging, Fertility-sparing, Chemotherapy, Molecular targeting agents

Background

Clear cell adenocarcinoma (CCC) is a distinct entity from other epithelial ovarian carcinomas (EOC). CCC is thought to arise from endometriosis or clear cell adenofibroma, however, the origin of serous cyst adenocarcinoma (SCA) is thought to be Mullerian epithelium derived from either ovarian surface epithelium or fallopian tube (endosalpingiosis). CCC has specific biological and clinical behavior, compared with other histological types. However, in the studies used as evidence for recommended treatment as standard treatment of EOC, most of the enrolled patients were not clear cell histology, and these study results do not provide a scientific rationale for CCC. In this review, we summarize the treatment of CCC.

Surgical treatment

The standard surgical treatment of patients with EOC is based on hysterectomy, bilateral salpingo-oophorectomy and partial omentectomy with peritoneal sampling and lymphadenectomy, and cytoreductive surgery is added especially for advanced cases. The surgical treatment of CCC is usually determined based on the guideline of EOC. In this section, we summarize the surgical treatment of CCC patients.

Surgical staging

It has been reported that the incidence of lymph node metastasis in stage I (pT1) EOC was approximately 5-

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Table 1 Rates of lymph node metastasis in early-staged clear cell carcinoma and serous adenocarcinoma

author	year	number of patients	pT stage	metastatic rate
clear cell carcinoma				
Di Re[2]	1989	11	pT1	9% (1/11)
Petru[3]	1994	2	pT1	0% (0/2)
Onda[4]	1996	16	pT1/2	31% (5/16)
Baiocchi[5]	1998	21	pT1	5% (1/21)
Suzuki[6]	2000	9	pT1	11% (1/9)
Sakuragi[7]	2000	23	pT1/2	17% (4/23)
Negishi[8]	2004	46	pT1	12% (5/42)
			pT2	75% (3/4)
Takano[9]	2006	173	pT1a	9% (3/36)
			pT1c	7% (7/99)
			pT2	13%(5/38)
Harter[10]	2007	7	pT1	0% (0/7)
Desteli[11]	2010	4	pT1	0% (0/4)
Nomura[12]	2010	36	pT1/2	6% (2/36)
Subtotal		**348**		**11%(37/348)**
Serous cystadenocarcinoma				
Di Re[2]	1989	40	pT1	28% (11/40)
Petru[3]	1994	21	pT1	38% (8/21)
Onda[4]	1996	21	pT1/2	33% (7/21)
Baiocchi[5]	1998	106	pT1	26% (27/106)
Suzuki[6]	2000	13	pT1	31% (4/13)
Sakuragi[7]	2000	25	pT1/2	8% (2/25)
Morice[13]	2003	26	pT1	31% (8/26)
Negishi[8]	2004	35	pT1	4% (1/24)
			pT2	36% (4/11)
Harter[10]	2007	13	pT1	15% (2/13)
Desteli[11]	2010	7	pT1	14% (1/7)
Nomura[12]	2010	12	pT1/2	50% (6/12)
Subtotal		**319**		**25%(81/319)**

20% [1-6]. Reported rates of lymph node metastasis in CCC and serous cystadenocarcinoma (SAC) were summarized in Table 1 [2-14]. From the results investigating a large number of CCC cases, retroperitoneal lymph node metastasis was observed in 9% in pTIa tumors, 7% in pTIc tumors, and 13% in pT2 tumors in CCC, which suggested that incidence of lymph node metastasis in CCC was lower than that of SAC [9]. Based on the subtotal of reported cases with pT1 and pT2 tumors, approximately one half incidence of lymph node metastasis in CCC in comparison with SAC was confirmed: 11% in CCC, and 25% in SAC.

Lymphadenectomy is so important to detect metastatic lymph nodes, as the patients with positive lymph nodes had poorer prognosis. However, the role of lymphadenectomy remains unclear based on the therapeutic aspect. Several authors reported that lymph node metastasis is independent prognostic factor for CCC [7,8,15]. Magazzino et al. analyzed 240 CCC retrospectively and reported as followed [15]: (1) Of 240 cases, 47.9% had lymphadenectomy and most of cases received platinum based chemotherapy after primary surgery. (2) The cases who received lymphadenectomy had longer progression-free survival (PFS) than the cases who had no lymphadenectomy in stage I/II, III/IV and all stage ($p = 0.0258$, $p = 0.00337$, $p = 0.0001$). (3) In advanced cases, lymphadenectomy prolonged the overall survival (OS). (4) In CCC, lymphadenectomy and clinical stage are independent prognostic factors by multivariate analysis. However, we reported that pN status showed only a marginal significance upon PFS and no significance upon OS based on the analysis of 199 CCC [16]. Other reports failed to show the usefulness of lymphadenectomy as prognostic factor [17,18]. Further examination will be required to confirm the role of lymphadenectomy for CCC.

In our studies, multivariate analysis revealed that peritoneal cytology status was independent prognostic factor for PFS ($p = 0.04$), but not for OS, and in addition, completion of surgical staging procedures was not a prognostic factor [16]. Higashi et al. analyzed 224 CCC patients with stage I and reported as followed [19]: (1) there was no significant difference in both OS and PFS of CCC between stage IA and IC (intraoperative capsule rupture), and the 5-year OS rate of stage IC(intraoperative capsule rupture) CCC patients was comparable to those with the non-CCC. (2) Stage IC CCC patients except for IC (intraoperative capsule rupture), such as positive ascites/washing and capsule surface involvement, had a poorer OS and PFS than those with IC (intraoperative capsule rupture). The results suggested stage I CCC cases other than intraoperative capsule rupture were at a considerable risk for recurrence and mortality.

Finally, the role of complete surgical staging still remains unclear for CCC. Several reports demonstrated that adjuvant chemotherapy had little impact on the survival of stage I CCC patients [16,20]. From these findings, complete surgical staging procedures are required at least to detect high-risk patients of recurrence; however, the extent of the surgery could not improve overall survival of CCC.

Cytoreductive surgery

Optimally cytoreduced patients of EOC were reported to show a significant survival benefit over those patients who are suboptimally debulked, and there is a significant survival advantage in patients who are able to be debulked to less than 1 cm of residual disease. Hoskins et al. reported that patients with clear cell and mucinous histology had poor outcome even when they had small

residual tumor after primary surgery [21]. We previously reported that there is no significant prognostic difference between the patients with the tumor diameter less than 1 cm and those with the tumor diameter more than 1 cm, and complete surgery is only the independent prognostic factor [9]. Kennedy et al. reported that among patients with advanced stage cancers (FIGO stages III and IV), CCC patients were more often optimally debulked than non-CCC patients (60% vs. 37%, p = 0.033) [22]. From these findings, the goal of primary surgical treatment for CCC may be complete resection.

Fertility-sparing surgery

Fertility-sparing surgery (FSS) for reproductive-age patients with EOC has been adopted for stage IA and non-clear cell histology grade 1 (G1)/grade2 (G2) according to the 2007 guidelines of the American College of Obstetrics and Gynecology (ACOG) and unilateral stage I tumor without dense adhesions showing favorable histology (ie, non-clear cell histology G1/G2) according to the 2008 guidelines of the European Society for Medical Oncology (ESMO). In Japan, stage IA tumor or unilateral stage IC tumor on the basis of intraoperative capsule rupture and favorable histology are candidate for FSS according to the 2010 guidelines of the Japan Society of Gynecologic Oncology (JSGO). These 3 guidelines commonly eliminate CCC for the candidate of FSS. In contrast, in the 2010 guidelines of the National Comprehensive Cancer Network (NCCN), a stage I patient with CCC is an acceptable candidate for FSS. For the patients to receive FSS, randomized study cannot be performed because of ethical aspect. In this review, we summarize the FSS for CCC based on the retrospective studies.

Schilder et al. demonstrated that no recurrence was observed among 5 patients with stage IC CCC who received FFS; however, the detail of stage or postoperative chemotherapy was not recorded [23]. Kajiyama et al. reported the clinical outcome of 10 patients with stage I CCC treated with FSS (IA:4, IC(intraoperative capsule rupture): 5, IC(positive for malignant ascites):1) and demonstrated as follow [24]: (1) Among 10 patients, 9 patients received chemotherapy after surgery, (2) one patient with IC(positive for malignant ascites) who received postoperative chemotherapy recurred. Sato et al. reported 30 patients with stage I CCC who received FFS and reported as follow [25]: (1) Among 15 IA cases, 9 cases received chemotherapy after surgery and no one recurred, (2)Among 15 IC patients, 11 patients received chemotherapy after surgery, and 2 patients (IC(intraoperative capsule rupture):2) recurred among 11 patients who received chemotherapy and 3 patients (IC(intraoperative capsule rupture):2, IC(positive for malignant ascites or surface capsule involvement):1) recurred among 4 patients who did not received chemotherapy. (3) Recurrent sites are residual ovary (n = 3), lymph node (n = 2), peritoneum (n = 2) and liver (n = 1). (4) The 5-year survival rate is 93.3%. These data are shown in Table 2.

We summarized Kajiyama's and Sato's reports in detail: (1) Among 19 patients, 12 patients received postoperative chemotherapy and no one recurred. (2) Among 21 IC patients, 17 patients received postoperative chemotherapy, and recurrent rate of IC(intraoperative capsule rupture) and IC(positive for malignant ascites or surface capsule involvement) are 25%(4/16) and 40%(2/5). (3) Among 17 IC patients who received postoperative chemotherapy, 3 (18%) patients recurred and among 4 IC patients who did not received chemotherapy, 3 (75%) patients recurred.

Recently, Kajiyama et al. also analyzed the OS of 16 patients with stage I CCC who underwent FSS and compared survival with 204 patients receiving radical surgery, or 64 patients with non-CCC undergoing FSS and demonstrated that patients with CCC who underwent FSS did not show a poorer survival than non-CCC patients who underwent FSS, or those at the corresponding stage with no CCC [26].

From these findings, CCC IA patient may be candidate for FFS and postoperative chemotherapy may be useful for CCC IC patient who received FFS.

Chemotherapeutic treatment

Clear cell carcinoma (CCC) is a quite unique ovarian tumor showing resistance to platinum-based chemotherapy. The effect of the gold standard therapy for ovarian carcinomas, combination with paclitaxel and carboplatin (TC), is not satisfactory for CCC. Irinotecan hydrochloride, a topoisomerase I inhibitor, is a candidate for the treatment for CCC. Irinotecan combined with cisplatin (CPT-P) has been recognized to have an activity no less than TC for CCC. A world-wide prospective clinical study to compare CPT-P and TC as the first-line chemotherapy for CCC, GCIG/JCOG (Gynecologic Cancer Intergroup/Japanese Gynecologic Oncology Group)

Table 2 Relapse rates of clear cell carcinoma patients who received FSS

stage	author	year	number of patients	relapse
Stage IA	Kajiyama [23]	2008	4	0% (0/4)
	Satoh [24]	2010	15	0% (0/15)
	total		19	0% (0/19)
Stage IC	Schilder [22]	2001	5	0% (0/5)
	Kajiyama [23]	2008	6	17% (1/6)
	Satoh [24]	2010	15	33% (5/15)
	total		26	23% (6/26)

3017, is now ongoing. Additionally, molecular-targeting agents are evaluated for advanced or recurrent CCC. We would discuss the chemotherapeutic regimens as primary or second-line therapy for CCC in this review.

Primary chemotherapy using cytotoxic agents

It has been implied that CCC of the ovary showed resistance to conventional platinum-based chemotherapy [27-29]. Recent studies have confirmed the evidence in the analysis of patients with measurable CCC. Objective response was observed in 11-27% with conventional platinum-based regimen, whereas patients with serous adenocarcinoma (SAC) subtype showed a significantly higher response rate of 73-81% [30-32]. A report showed survival benefit of conventional chemotherapy with paclitaxel and platinum after complete surgery in CCC patients [33]. However, the result from large series of CCC patients treated with paclitaxel and platinum showed no survival benefit compared with conventional platinum-based chemotherapy in both early and advanced cases [9]. The results suggested that TC therapy, which is commonly used for ovarian carcinoma, is not effective enough for CCC patients. Reported response rates of primary therapy for CCC are summarized in Table 3 [9,29-33].

Irinotecan hydrochloride, a semisynthetic derivative of camptothecin, has additive and synergic effects in combination with cisplatin *in vitro* [34,35]. The combination therapy with irinotecan hydrochloride and cisplatin (CPT-P) was reported to be effective for patients with various solid tumors. Especially, a large clinical trial revealed that CPT-P had significant activity for extensive small-cell lung cancer [36]. Additionally, CPT-P had been reported to be effective in first-line and second-line chemotherapy for the treatment of CCC of ovary [37,38]. A large retrospective analysis indicated that CPT-P had a potential therapeutic effect at least no less than TC therapy [39]. A phase II study (JGOG3014) to compare CPT-P and TC for first-line treatment for CCC was conducted. The study revealed that completion rate of six cycles and five-year progression-free survival was similar in both arms [40]. Interesting to note, in the patients with residual tumor less than 2 cm, overall survival was marginally improved in CPT-P group in comparison with TC group ($p = 0.056$). Subsequently, a phase III randomized study to compare CPT-P and TC as adjuvant chemotherapy for CCC is on-going (GCIG/JGOG3017) [41]. The winner regimen will be the first regimen for histologically individualized therapy for ovarian cancers.

Table 3 Response rates of primary chemotherapy for clear cell carcinoma

regimen	author	year	response/ Number of patients, response rate
Conventional Platinum-based	Goff [28]	1996	1/6, 17%
	Sugiyama [29]	2000	3/27, 11%
	Ho [30]	2004	4/15, 27%
	Takano [9]	2006	5/30, 17%
Taxane-Platinum	Enomoto [31]	2003	2/9, 22%
	Ho [30]	2004	9/16, 56%
	Utsunomiya [32]	2006	8/15, 53%
	Takano [9]	2006	9/28, 32%
Irinotecan-cisplatin	Takano [9]	2006	3/10, 30%

Another issue concerning chemotherapy for CCC is adjuvant therapy for patients with stage I disease. CCC is regarded as grade 3 tumor, and clinical guidelines recommend adjuvant chemotherapy for all patients with CCC, even at stage Ia. A large retrospective analysis of stage I CCC revealed that there were no statistical differences of progression-free survival (PFS) and overall survival (OS) between patients with chemotherapy and without chemotherapy [16]. Also, multivariate analysis showed that peritoneal cytology status ($p = 0.02$) and pT status ($p = 0.04$) were independent prognostic factors for PFS, however, adjuvant chemotherapy was not a prognostic factor ($p = 0.80$). The results suggested adjuvant chemotherapy had little impact upon survival of stage I CCC patients. Further strategy, such as a molecular targeting agent, is needed to improve survival of CCC, especially cases with positive peritoneal washing.

Second-line chemotherapy for CCC

In a large series of platinum-sensitive relapsed ovarian tumors including all histological subtypes, overall response was 54% of the patients treated with the conventional platinum-based chemotherapy, and 66% of the cases treated with paclitaxel plus platinum chemotherapy [42]. In the platinum-resistant tumors, however, response rate using anti-cancer agents usually range from 25 to 30% [43]. In the second-line or salvage settings, the response rate for recurrent or refractory CCC was extremely lower than that for other histological tumors: even in the patients with platinum-sensitive CCC disease, the response rate reported was lower than 10% [44,45]. So, we have summarized reported cases that achieved objective response (Table 4) [30,33,44-48].

Recently, single agent gemcitabine could be a candidate for salvage therapy for CCC, as the authors suggested [44,48]. Other regimens that showed objective response included irinotecan/platinum, etoposide/platinum, and paclitaxel/carboplatin; however, the efficacy was limited with progression-free interval approximately 6 months. Despite importance of response, it would be more important to monitor if adverse effects of chemotherapy worsen quality of life of the patients. Among

Table 4 Response rates of salvage chemotherapy for recurrent or refractory clear cell carcinoma

regimen	author	year	response/ number of patients, response rate
Megestrol acetate	Walailak [45]	2001	2/10, 20%
Cyclopshosphamide+ cisplatin	Takano [46]	2008	1/9, 11%
Irinotecan+Platinum	Sugiyama [29]	1998	1/3, 33%
	Takano [46]	2008	2/15, 13%
Etoposide+Platinum	Takano [46]	2008	2/13, 15%
Paclitaxel+Carboplatin	Utsunomiya [32]	2006	3/13, 23%
	Crotzer [43]	2007	2/7, 29%
Gemcitbine	Crotzer [43]	2007	1/9, 11%
	Yoshino [47]	2012	1/5, 20%
Docetaxel+Irinotecan	Yoshino [47]	2012	1/11, 9%
Temsirolimus	Takano [46]	2011	1/5, 20%

these reports, the longest progression-period of 14 months was obtained by Temsirolimus [47]. The observed response duration was surprisingly longer than those obtained by any cytotoxic agents so far with no serious toxicities. The report encouraged us to investigate another chemotherapeutic strategy for CCC.

From the reported cases, however, it could be concluded that CCC is a potentially extremely chemo-resistant tumor against cytotoxic agents, especially in recurrent or refractory settings. Another strategy including molecular targeting agents might be needed for the treatment of these tumors.

Incorporation of molecular targeting agents for the treatment of CCC

In the aspects of molecular characteristics as well as clinical behavior, it is hypothesized that CCC belongs to a different entity from other histological subtypes of ovarian carcinoma. First of all, the incidences of p53 mutation and p53 overexpression were much less frequent in CCC than in other histologic types of epithelial ovarian cancer [49,50]. On the other hand, mutation of p53 gene was quite frequent in serous subtype of ovarian cancers, and most of the alterations were missense mutations [51]. In addition to p53 status, CCC has a quite unique expression pattern of several molecules. Glutathione peroxidase 3 (GPX3) was found at levels 30-fold higher on average in CCC compared with the other ovarian cancer subtypes through studies with cDNA arrays and serial analysis of gene expression [52]. Elevated expression of GPX3 might contribute to chemoresistance phenotype, which is often observed in the patients with CCC. Another investigation using oligonucleotide microarrays reported that glutaredoxin (GLRX) and superoxide dismutase 2 (SOD2), in addition to GPX3, were highly expressed in clear cell type ovarian cancer, suggesting that high levels of these proteins relating with antioxidant function render CCC to be more resistant to chemotherapy [53,54].

Further, a report using oligonucleotide probe arrays showed that a transcription factor, hepatocyte nuclear factor-1 (HNF-1) was upregulated in CCC cell lines [55]. Overexpression of HNF-1 was confirmed by immunohistological staining of clinical samples. Further, overexpression of HNF-1 was observed in the specimens of borderline clear cell tumor and benign clear cell tumor [56]. The expression of HNF-1 was detected in not only atypical endometrial tissue, but also in endometriosis with degenerative and regenerative changes, suggesting that early differentiation into the clear cell lineage takes place in the endometriotic epithelium, and HNF-1 contributes to carcinogenesis of CCC.

Recently, immunohistochemical analysis showed that hypoxia-inducible factor 1 alpha (HIF-1alpha) expression levels were significantly higher in CCC than in other histological types of ovarian cancers [57]. Upstream target of HIF-1alpha, mammalian target of rapamycin (mTOR), was also reported to be up regulated in CCC [58,59], which was selected for molecular target of CCC.

There are two international collaborating studies led by Gynecologic Oncology Group (GOG) to evaluate efficacy of molecular targeting agents for CCC of the ovary [60,61]. It is true that there existed super-responders against molecular targeting agents in the patients with CCC. Consequently, further studies to evaluate these new drugs should include biomarker analysis to predict response or adverse effect for clinical application.

Conclusions

CCC has unique characteristics among ovarian cancers. We have to deal with the tumor using completely different techniques of treatment modality in terms with surgery and chemotherapy. Especially, we have to focus on histology-specific features of molecular pattern. We hope the day will come when CCC tumors would be easily handled by the selection of effective surgery and chemotherapy including molecular targeting agents.

Abbreviations

CCC: Clear cell adenocarcinoma; SAC: Serous cyst adenocarcinoma; EOC: Epithelial ovarian carcinomas; PFS: Progression free survival; OS: Overall survival; FSS: Fertility-sparing surgery; ACOG: American college of obstetrics and gynecology; ESMO: European society for medical oncology; JSGO: Japan society of gynecologic oncology; NCCN: National comprehensive cancer network; GCIG: Gynecologic cancer intergroup; JCOG: Japanese gynecologic oncology group; CPT-P: Irinotecan hydrochloride + cisplatin; TC: Paclitaxel + carboplatin; GPX3: Glutathione peroxidase 3; GLRX: Glutaredoxin; SOD2: Superoxide dismutase 2; HNF-1: Hepatocyte nuclear factor-1; HIF-1: Hypoxia-inducible factor 1; mTOR: Mammalian target of rapamycin; GOG: Gynecologic oncology group.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions
Dr Takano and Dr Tsuda wrote the manuscript. Dr Takano, Dr Tsuda, and Dr Sugiyama approved it. All authors read and approved the final manuscript.

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Enhanced Expression of Sulfatide, a Sulfated Glycolipid, in Well-Differentiated Endometrial Adenocarcinoma

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Objectives: It is well known that a poorly differentiated endometrial adenocarcinoma shows more rapid progression and a worse response to therapy than a well-differentiated endometrial adenocarcinoma. Qualitative and quantitative changes of cell surface glycolipids occur during neoplastic transformation. Sulfatide is one of the sulfated glycolipids in the cell membrane that may have an important role in various functions such as cell adhesion. To examine the molecular background of the morphological and biological features of well-differentiated and poorly differentiated cancer, we measured the levels of lipids, especially glycolipids, in tumor tissues from patients with endometrial carcinoma.

Materials and Methods: We determined the composition of lipids and glycolipids in tumor tissues, investigated glycosyltransferase messenger RNA expression by the reverse transcription-polymerase chain reaction, and assessed the localization of galactosylceramide sulfotransferase (an enzyme involved in sulfatide biosynthesis) by immunohistochemical staining.

Results: No significant differences were observed between well-differentiated and poorly differentiated cancer with respect to the levels of cholesterol ester, cholesterol, phospholipids, cholesterol sulfate, ceramides, neutral glycolipids of the globo series, and GM3 ganglioside. However, the amount of sulfatides in well-differentiated tumors was significantly greater than that in poorly differentiated tumors, which was confirmed by thin-layer chromatography and immunostaining with a monoclonal antisulfatide antibody. Altered expression of sulfatide was found to be secondary to a change of galactosylceramide sulfotransferase messenger RNA expression. Immunohistochemical staining revealed that galactosylceramide sulfotransferase expression was characteristically observed in glandular areas but not in solid areas.

Conclusion: These findings suggest that sulfatide contributes to the well-differentiated phenotype of endometrial adenocarcinoma and that it is being expressed in normal uterine endometrium at sites of gland formation during the luteal phase, as we have previously reported.

Key Words: Endometrioid adenocarcinoma, Glandular differentiation, Sulfatide, Carbohydrate sulfation, Tumor grade

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A common phenotypic change associated with the development of malignancy is a dramatic alteration of cellular glycosylation. The significant correlation between aberrant glycosylation of primary tumors and the prognosis has stimulated interest in identifying the biological functions of glycolipids. Tumor cell carbohydrates have been shown to participate in cell-cell interactions, and glycolipids have been identified as potential effectors of signal transduction. Sulfatides are a class of sulfate-containing glycolipids that are distributed in various tissues, including the brain, kidney, and gastrointestinal tract. Biosynthesis of sulfatides requires the transfer of sulfate to the glycolipid moiety, and this reaction is catalyzed by galactosylceramide sulfotransferase (GcST). The physiological functions of sulfatides have been investigated in mice lacking the *GcST* gene, which show complete absence of sulfatides,[1,2] along with neurological disorders and arrest of spermatogenesis. Several studies have provided evidence that sulfatides expressed by different cells mediate interactions with various proteins, such as laminin, thrombospondin, amphoterin, selectins, galectin, and hepatocyte growth factor. Some of these proteins are adhesion molecules involved in cell-cell and cell–extracellular matrix interactions.

A relationship has been reported between the prognosis of endometrial carcinoma and tumor differentiation (ie, gland formation). A poorly differentiated endometrial adenocarcinoma shows more rapid progression and is less responsive to therapy than a well-differentiated adenocarcinoma, resulting in a worse prognosis. Diagnosis of tumor differentiation is always done by pathologists, and the tumor grade that they assign might be the single most important prognostic factor for endometrial cancer. However, little basic research has been done on the process of transformation of endometrial cancer to the well-differentiated or poorly differentiated phenotype. Understanding the mechanism involved in the differentiation of endometrial cancer might lead to new therapy that induces tumor differentiation. Our previous studies have demonstrated that sulfatide is expressed by the endometrium during the secretory phase rather than during the proliferative phase, that is, sulfatide expression declines during the proliferative phase controlled by estrogen.[3] We have also analyzed the composition of sulfated glycolipids expressed by cultured cell lines derived from cervical cancer, ovarian cancer, and endometrial cancer and have demonstrated that sulfated glycolipids are particularly expressed by cells originating from endometrial cancer.[4–6] These results suggest that sulfated glycolipids are involved in endometrial carcinogenesis and in the differentiation of the endometrium under the influence of sex steroid hormones.

Accordingly, the objectives of the present study were to compare the expression of glycolipids between well-differentiated and poorly differentiated adenocarcinoma of the endometrium and to investigate the role of sulfatide in morphological differentiation (gland formation), which is the most important prognostic factor for endometrial cancer.

MATERIALS AND METHODS

Materials

Glycolipids from various sources were purified in our laboratory, including GlcCer, LacCer, Gb_3Cer, Gb_4Cer, and GM3 from human erythrocytes, and GalCer, sulfatide, and sphingomyelin from bovine brain. Ceramides were prepared by treatment of sphingomyelin with *Clostridium perfringens* phospholipase C, which was kindly provided by Dr M. Kitamura (National Institute of Infectious Disease, Tokyo, Japan). Cholesterol and cholesterol 3-sulfate (CS) were purchased from Sigma (St. Louis, MO). Dioleoyl phosphatidyl ethanolamine, phosphatidylglycerol, phosphatidylcholine, and phosphatidylserine were provided by Aswell Co (Osaka, Japan). A monoclonal antibody (TCS-1) targeting I^3SO_3-GalCer (sulfatide) and II^3SO_3-LacCer was generated in our laboratory by immunization of mice with sulfatide and *Salmonella Minnesota* as the adjuvant and was proven to exhibit specific reactivity with SO_3-3Gal. A monoclonal antibody targeting Le(y) (NCC-ST-433) was kindly provided by Dr S. Hirohashi (National Cancer Center, Tokyo, Japan). Monoclonal antibodies directed against GM3 (M2590), GD3 (S2-566), and anti–sialyl Le(a) (2D3) were obtained from Seikagaku (Tokyo, Japan). Mouse antihuman Lewis(y) monoclonal antibody (clone A70-C/C8) and rabbit antihuman-GcST monoclonal antibody (anti-GAL3ST1 antibody) were purchased from Abcam (Cambridge, UK).

Tumor Tissues

Tumor tissues were obtained from the Department of Obstetrics and Gynecology at Tokai University Hospital. Written informed consent to use tumor specimens for this study was obtained from all subjects, and the experimental protocol was approved by the hospital Ethics Committee. Histological classification of the tumors was performed according to the criteria of the International Federation of Gynecology and Obstetrics. A total of 19 endometrial adenocarcinomas were studied; 7 well-differentiated tumors (G1 samples 1–7) and 5 poorly differentiated tumors (G3 samples 8–12) were used for biochemical analysis and thin-layer chromatography (TLC) immunostaining, whereas 7 moderately differentiated tumors (G2 samples 13–19) were used for immunohistochemical examination. Tumor differentiation was diagnosed histologically according to the amount of glandular and solid areas in the cancerous tissue. Because the diagnosis of moderately differentiated adenocarcinoma tends to vary among pathologists, the G1 and G3 samples were used for biochemical analysis, whereas G2 samples were only used for immunohistochemical examination to detect the localization

of GcST in the glandular and solid areas. Tumor samples were immediately stored at −70°C until use. For histological examination, tissues were fixed in formalin and embedded in paraffin, after which sections 4 μm thick were cut and stained with hematoxylin and eosin.

Separation and Quantitation of Lipids

After lyophilization of tumor tissues, total lipids were extracted from the lyophilized powder with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1 by volume). Cholesterol sulfate, ceramides, and phospholipids were developed on TLC plates with cholesterol/methanol/acetone/acetic acid/water (8:2:4:2:1 by volume) chloroform/methanol/acetic acid (94:1:5 by volume) and chloroform/methanol/water (65:35:8 by volume), respectively. The concentrations of these lipids were determined by TLC densitometry at an analytical wavelength of 500 nm after visualization with cupric acetate-phosphoric acid. Then the lipid extracts were fractionated into neutral and acidic lipids on a DEAE-Sephadex column (A-25, acetate form; Pharmacia, Uppsala, Sweden). Preparation of neutral and acidic glycolipids was carried out as described previously.[7–9] The neutral glycolipids were separated from the unabsorbed neutral lipid fraction by acetylation, separation of the acetylated derivatives, deacetylation, and desalting, whereas the acidic glycolipids were prepared from the absorbed acidic lipid fraction by cleavage of the ester-containing lipids and subsequent dialysis. The acidic and neutral glycolipids thus obtained were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10 by volume) and chloroform/methanol/water (65:35:8 by volume), and then were visualized with resorcinol-HCl and orcinol-H_2SO_4, respectively. The density of spots was determined at an analytical wavelength of 580 nm for resorcinol-HCl-positive spots and 420 nm for orcinol-H_2SO_4-positive spots, respectively, using a dual-wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto, Japan). Standard glycolipids, which were *N*-stearoyl derivatives of GalCer, LacCer, Gb_3Cer, and GM3 (0.1–1.5 μg), were developed on the same TLC plates to allow creation of standard curves for quantitation.

TLC Immunostaining

The total lipid extracts were applied to plastic-coated TLC plates, which were developed as described previously. Each plate was incubated with a blocking buffer (1% polyvinylpyrrolidone [PVP] and 1% ovalbumin in phosphate-buffered saline [PBS]) at 4°C overnight and then with antibodies in 3% PVP in PBS at 37°C for 2 hours. The plates were subsequently washed 5 times with 0.1% Tween 20 in PBS, and the antibodies bound to the plates were detected using peroxidase-conjugated antimouse and antihuman (IgG and M) antibodies (Cappel Laboratories, Cochranville, PA), diluted 1:1000 (by volume) with 3% PVP in PBS. Reaction products were detected with H_2O_2 and 4-chloro-1-naphthol, as described previously.[6]

Reverse Transcriptase Polymerase Chain Reaction Analysis

Total RNA was extracted from tumor tissues with Isogen (Wako, Tokyo, Japan) and was reacted with reverse transcriptase (M-MuLV; Takara, Kyoto, Japan) and random primers to obtain complementary DNA. This was then subjected to polymerase chain reaction (PCR) under the conditions of 35 cycles at 95°C for 15 seconds, 52°C to 64°C for 30 seconds, and 72°C for 40 seconds. The following primers were used: ceramide glucosyltransferase (GlcCerT, NM003358), CAAAACTCTGGCTCATATTC (sense), and ATATTGTCATGGA TTCGCGG (antisense); ceramide galactosyltransferase (GalCerT, NM003360), CTCTCTGAAGGCA GAGACATCGCC (sense), and CATCCACAGGCTGGACCCA TGAAC (antisense); LacCer sialyltransferase (GM3T, AB018356), ATTTGAGCACAGGTATAGC (sense), and GATGTCAAAGG CAGTCTCT (antisense); cholesterol sulfotransferase (SULT2B1b, U92315), GGCTTGTGGGACACCTATGAAGATGACATC (sense), and GCTCCTCGTAGGTGATAAATAGG (antisense); galactosylceramide sulfotransferase (GcST, NM_004861), CTAC TTCAAGCTCAACGCCC (sense), and CTTGAGGTTGTAGC CCAGGA (antisense); and GM3 *N*-acetylgalactosaminyltransferase (GM2T, M83651), AGAAACAAGTCCGAGCTATT (sense), and AAGGGCATGAGATAGTGTTC (antisense). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. The PCR products were subjected to electrophoresis on 1.5% agarose gel, stained with ethidium bromide, and then examined under UV light.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue blocks were cut into 4-μm-thick sections for immunohistochemical examination and for hematoxylin and eosin staining. Immunohistochemical staining was carried out with an avidin-biotin-peroxidase system (Vectastain ABC kit, Vector Lab, CA), as reported previously.[10]

Statistical Analysis

SPSS version 16.0 software (SPSS Inc, Chicago, IL) was used for statistical analysis. The Student *t* test was used to compare data between 2 groups, and $P < 0.05$ was considered statistically significant.

RESULTS

Neutral Lipids and Phospholipids in Endometrial Carcinoma

Table 1 shows the concentrations (μg/mg dry weight) of major glycerophospholipids, including phosphatidylcholine plus phosphatidylserine, phosphatidyglycerol, and phosphatidylethanolamine. There were no significant differences of these lipids between the well-differentiated tumors and the poorly differentiated tumors. There were also no differences of cholesterol and sphingomyelin. Thin-layer chromatography of lipid extracts for ceramide species revealed that no ceramides with hydroxyl fatty acids were detected in either the well-differentiated tumors or the poorly differentiated tumors (data not shown).

Neutral Glycolipids in Endometrial Carcinoma

As shown in Table 1, neutral glycolipids belonging to the globo series were predominant in both the well-differentiated

TABLE 1. Lipid compositions (μg/mg dry weight) in endometrial adenocarcinoma tissues of well-and poorly differentiated types

Case	Tumor Grade	Glycerophospholipids					Neutral Glycolipids				Acidic Lipids		
		Cho	PE	PG	PC/PS	SM	Ceramide	GlcCer	LacCer	Gb3	Gb4	Sulfatide	GM3	CS
1	G1	12.9	7.94	0.88	6.95	1.04	1.03	0.44	0.92	0.69	0.49	0.12	0.31	0.06
2	G1	10.1	7.69	0.99	6.58	1.86	1.09	0.85	0.79	1.22	1.85	0.21	0.72	0.07
3	G1	10.2	4.41	0.68	4.28	1.49	1.19	0.88	0.74	0.28	0.3	0.07	0.18	0.07
4	G1	9.4	6.3	0.94	6.42	1.7	1.07	0.6	1.42	1.16	0.49	0.11	0.74	0.08
5	G1	7.8	5.83	0.86	6.3	1.67	1.43	0.55	0.83	1.26	0.73	0.16	0.31	0.06
6	G1	8.6	5.77	0.83	5.46	1.29	1.16	0.49	1.44	0.96	0.92	0.09	0.6	0.02
7	G1	8.3	7.32	1.06	6.21	1.69	0.99	0.84	1.72	1.25	0.87	0.34	0.44	ND
8	G3	11.4	9.49	1.08	6.55	1.85	1.48	0.48	1.1	1.75	1.21	ND	0.66	0.08
9	G3	14.8	7.82	0.53	7.17	2.68	1.86	0.78	1.28	1.26	0.98	0.04	0.59	0.02
10	G3	11.3	7.6	0.64	3.26	1.56	0.69	0.47	1.02	1.2	1.19	ND	0.67	0.08
11	G3	9.8	8.13	1.2	6.92	2.38	0.83	0.49	0.75	0.29	0.33	ND	1.15	ND
12	G3	11	7.76	0.71	6.76	2.63	0.49	0.47	1.21	0.81	0.75	ND	0.64	0.01

Cho, cholesterol; G1, well differentiated; G3, poorly differentiated; ND, not detected; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; SM, sphingomyelin.

tumors and the poorly differentiated tumors. There were no differentiation-associated characteristic patterns of these neutral glycolipids.

Acidic Lipids in Endometrial Carcinoma

Cholesterol 3-sulfate and sulfated and sialylated glycolipids were detected in the acidic lipid fraction. As shown in Figure 1, both the well-differentiated tumors and the poorly differentiated tumors contained 2 major glycolipids, which were sulfatide and GM3. Interestingly, the amount (μg/mg dry weight) of sulfatides in the well-differentiated tumors was significantly greater than that in the poorly differentiated tumors ($P < 0.05$; Table 1). Although CS was detected in all tumor tissues, the actual levels were low (<0.08-μg/mg dry tissue; Table 1).

TLC Immunostaining of Acidic Lipids in Endometrial Carcinoma

Thin-layer chromatography immunostaining was performed for sulfatide, GM3, and GD3 to confirm the distribution of acidic glycolipids in endometrial carcinoma. As shown in Figure 2, the amount of sulfatide (μg/mg dry weight) in the well-differentiated tumors was markedly greater than that in the poorly differentiated tumors. Specimen number 7, a well-differentiated tumor, was found to contain lactosylsulfatide and sulfatide. In contrast, GM3 and GD3 were detected in both the well-differentiated tumors and the poorly differentiated tumors (Fig. 3). In addition, carbohydrate antigen, Lewis(y), and sialyl Lewis(a) were also detected in both types of tumor.

RT-PCR of Glycosyltransferase Gene Expression

To clarify the genetic background of the tumor glycolipid components, expression of glycosyltransferase genes was examined by reverse transcriptase PCR (RT-PCR; Fig. 3). When their expression was compared with that of glyceraldehyde 3-phosphate dehydrogenase (the control housekeeping gene), some genes were positively correlated with the

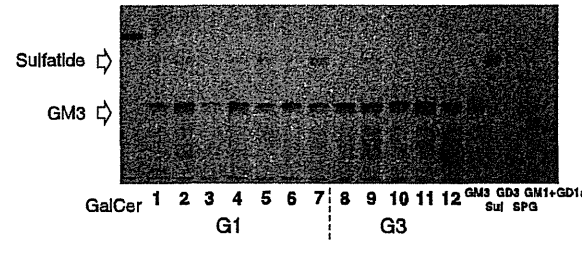

FIGURE 1. Acidic glycolipids in well-differentiated (G1 samples 1–7) and poorly differentiated (G3 samples 8–12) human endometrial adenocarcinoma acidic lipid extracts from well-differentiated and poorly differentiated endometrial adenocarcinomas (2-mg dry tissue weight) were developed on TLC plates with chloroform/methanol/water (55:45:10, vol/vol) and were detected with orcinol-H_2SO_4 reagent. GalCer, galactosylceramide; SPG, sialylparagloboside; Sul, sulfatide.

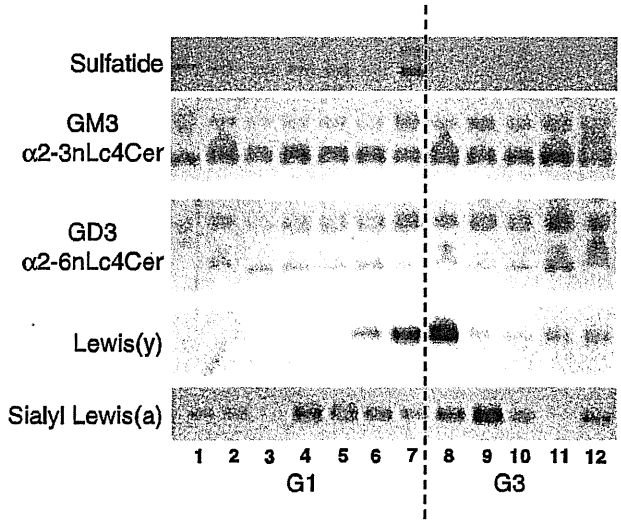

FIGURE 2. Thin-layer chromatography immunostaining of total lipid extracts from well-differentiated and poorly differentiated endometrial adenocarcinomas. Total lipids (0.2-mg dry tissue weight) were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, vol/vol) and were detected with antibodies directed against sulfatide, GM3, GD3, Lewis (y), and Sialyl Lewis (a).

tissue levels of certain lipids. For example, relatively low expression of the *SULT2b1* gene in specimens 9 and 11 corresponded with no detection of CS in these specimens (Table 1 and Fig. 3). In addition, GM3T gene expression was approximately related to the amount of GM3 in both the well-differentiated tumors and the poorly differentiated tumors (Figs. 2, 3). In addition, there was lower GcST gene expression in specimens 8 to 12 than in specimens 1 to 7, in which sulfatide levels were higher (Figs. 1, 3). Finally, GcST messenger RNA expression was stronger in the well-differentiated tumors than in the poorly differentiated tumors.

Immunohistochemistry for GcST

As shown in Figure 4A, a positive reaction was most frequently observed in the glandular areas of moderately differentiated adenocarcinoma, whereas a positive reaction was not seen in solid areas (Fig. 4B). The stromal components were almost entirely negative.

DISCUSSION

In our previous study, sulfatide expression was observed in the normal endometrium during the progesterone-dominant secretory phase rather than the estrogen-dominant proliferative phase.[3] In the present study, sulfatide expression was more prominent in well-differentiated endometrial cancer compared with poorly differentiated cancer, and the amount of sulfatide in the well-differentiated endometrial adenocarcinoma was similar to that in normal secretory endometrium.[3] Estrogen priming occurs in the normal endometrium, after which endometrial differentiation is triggered during the secretory phase by progesterone released from the ovary after ovulation. Expression of sulfatide is enhanced at this time owing to induction of GcST. Because sulfatide is expressed during the secretory phase, it is presumed to be involved in endometrial functions such as implantation. Well-differentiated endometrial cancer is induced by prolonged exposure to estrogen and expression of sulfatide is thought to be enhanced through induction of GcST by estrogen in patients with endometrial cancer, although this mechanism is opposite to the presumed effect of estrogen on GcST in the normal endometrium. Thus, sulfatide might be expressed at the functional stage of glandular formation in both endometrial cancer and the normal endometrium. Our group has also studied ovarian tumors (including mucinous cystadenocarcinoma, serous cystadenocarcinoma, and clear cell adenocarcinoma), and we have found that mucinous cystadenocarcinoma expresses sulfatide, although it is hardly detected in the normal ovary and is found in less than 40% of other ovarian tumors.[9] With respect to gland formation and mucus secretion, sulfatide might have a special role in gynecological adenocarcinomas. However, expression of sulfatide might be regulated differently among the normal endometrium, endometrial cancer, and mucinous cystadenocarcinoma of the ovary. Therefore, further analysis focusing on the substrates (galactosylceramide and 3′-phosphoadenosine- 5′-phosphosulfate), the synthesizing enzyme (GcST), and the degrading enzyme (arylsulfatase A) is needed, and such investigations might help to elucidate the functions of sulfatide in the normal endometrium and endometrial cancer.

As stated before, histological differentiation is an important determinant of the aggressiveness of endometrial cancer. In fact, preservation of the uterus and fertility-preserving therapy that uses high-dose progesterone or chemotherapy alone without surgical excision have recently been tried in patients with stage IA endometrial cancer who have well-differentiated

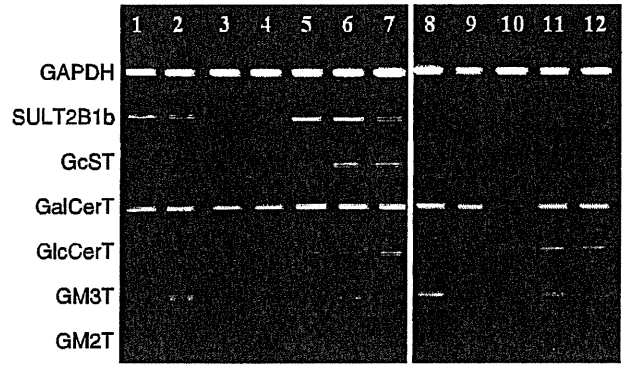

FIGURE 3. Reverse transcriptase polymerase chain reaction analysis of glycosyltransferase and sulfotransferase messenger RNA gene expression in well-differentiated and poorly differentiated endometrial adenocarcinomas of humans. GalCerT, ceramide galactosyltransferase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GlcCerT, ceramide glucosyltransferase; GM2T, LacCer *N*-acetylgalactosaminetransferase; GM3T, LacCer sialyltransferase; SULT2B1b, cholesterol sulfotransferase.

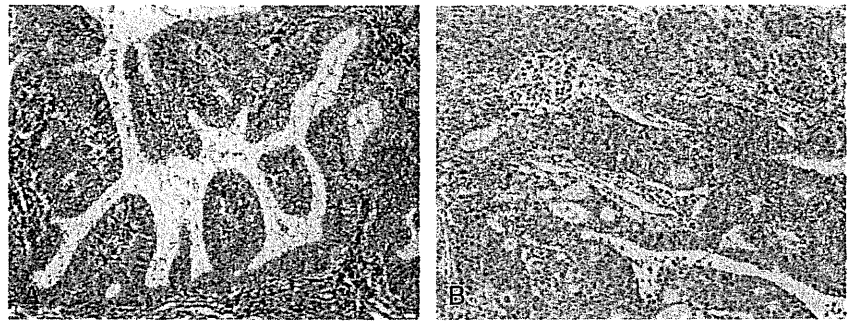

FIGURE 4. A, B, Immunohistochemistry of galactosylceramide sulfotransferase (GcST) protein in moderately differentiated adenocarcinoma. A positive reaction was frequently observed in the glandular areas (A). There was no positive reaction in the solid areas (B).

tumors without muscle invasion.[11] It is thus interesting to investigate the mechanism and significance of enhanced sulfatide expression in the normal endometrium during the secretory phase of the menstrual cycle and in well-differentiated endometrial cancer. If poorly differentiated adenocarcinoma with its poor prognosis could be transformed into well-differentiated adenocarcinoma by induction of differentiation, such a reduction of tumor aggressiveness might lead to improvement of the prognosis. With this objective, we have previously performed differentiation of cultured cells derived from poorly differentiated endometrial cancer in 3-dimensional type I collagen gel culture, as well as induction of gland formation.[6] These studies suggested the significance of cholesterol sulfate and sulfatide expression. All of the cells expressed sulfatide, whereas differentiation could only be induced in cells that did not express cholesterol sulfate. These results suggested the hypothesis that gland formation (differentiation of adenocarcinoma) occurred through an interaction between the type I collagen substrate of our cultures and cellular glycolipids containing sulfate groups. An increase in the expression of cholesterol sulfate on the cell membrane would mask glycolipids containing sulfate groups and weaken the interaction with type I collagen, leading to the poorly differentiated phenotype. If it were possible to induce the differentiation of poorly differentiated endometrial cancer in vivo, the prognosis of patients might be improved. Based on the results obtained in the present study, we plan to further investigate in vivo induction of the differentiation of endometrial cancer cells to develop differentiation inducers for clinical use.

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Long Term Prognostic Implications of Expression of Glucose Transporter-1 and Hexokinase II in Patients with Stage I Uterine Leiomyosarcoma

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Abstract

Many malignant epithelial tumors show increased expression of glucose transporter-1 (GLUT-1) and hexokinase II (HK-II), both of which are involved in glucose metabolism. GLUT-1 levels are often correlated with prognosis in these tumors. The current retrospective study was conducted to evaluate the importance of GLUT-1 and HK-II expression in leiomyosarcoma (LMS), a malignant uterine non-epithelial tumor with a poor prognosis. The subjects were 23 patients with stage I LMS. Expression of GLUT-1 and HK-II was evaluated immunohistochemically in samples removed surgically, and the MIB-1 index was evaluated as a measure of cell proliferation. The association of these results with prognosis was examined. Twenty samples of leiomyoma (LOM), a benign non-epithelial tumor, were used as controls. Immunohistochemical expression was defined as negative staining (–), weak to sporadic staining (1+), and strong staining (2+) per microscopic field, respectively. Malignancy was evaluated in 2000 cells and the MIB-1 index was calculated. Overall survival for LMS was estimated using the Kaplan-Meier method. Of the LMS cases, 12 were GLUT-1-positive (52.2%; 2+: 2, 1+: 10) and 15 were HK-II-positive (65.2%; 2+: 1, 1+: 14). GLUT-1 expression in LMS was significantly correlated with the MIB1 index. The 10-year survival rates were 90.9% and 58.3% in GLUT-1-negative and GLUT-1-positive cases, respectively, and 75.0% and 73.3% in HK-II-positive and HK-II-negative cases, respectively. GLUT-1 expression was significantly correlated with prognosis. Cases of stage I LMS showed a significant correlation between the expression level of GLUT-1 and the MIB-1 index, an indicator of malignancy. GLUT-1-negative cases had a better prognosis than GLUT-1-positive cases, suggesting that GLUT-1 expression is an effective prognostic marker.

Keywords: uterine leiomyosarcoma, immunoexpression, glucose transporter-1, hexokinase II

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I. Introduction

Leiomyosarcoma (LMS) is a malignant uterine non-epithelial tumor that accounts for 1% to 3% of all malignant tumors in women. LMS has a poor prognosis, since the primary tumor is likely to undergo recurrence and metastasis [7]. Tissue necrosis and higher mitotic rates are important indicators for malignancy and prognosis [18, 29, 40]. More than 80% of cases of stage III LMS show recurrence and metastasis and the 5-year survival rate in cases of stages II–IV is approximately 8%, indicating an extremely poor prognosis [9, 33]. Surgery is the first option for LMS treatment; however, even if LMS is in the early stage and can be completely removed, distant metastasis to the lung often occurs and results in a poor long-term prognosis [22]. Radiotherapy and combination chemotherapy with doxorubicin have also been used for LMS, but treatment outcomes remain poor [22, 34].

Many malignant epithelial tumors show increased glucose uptake [42]. Expression of glucose transporter-1 (GLUT-1) is often increased in malignant hypoxic cells and hexokinase II (HK-II) expression also increases. This causes resistance to radiotherapy and chemotherapy and enhanced recurrence and metastasis, which underlie the close relationship of GLUT-1 expression with prognosis [2, 10, 15, 28, 37]. In a clinicopathologic study of epithelial ovarian cancer, we found increased GLUT-1 expression and strong expression of hypoxia inducible factor-1 (HIF-1α), with a clear increase in glucose uptake.

Similarly, high expression levels of HIF-1α and GLUT-1 have been shown in clear cell carcinoma, which also has a poor prognosis and is common in Japanese patients [16, 43]. Thus, the current study was performed to examine expression of GLUT-1 and HK-II and the relationship of these data with the long term prognosis of LMS, which has not been examined in previous studies.

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II. Materials and Methods

Patients and treatments

The subjects were 23 patients (mean age: 51.5 years old; range: 35–70 years old) with clinical stage I LMS who underwent hysterectomy between March 1987 and May 2005 in our hospital. Adjuvant chemotherapy were performed in 14 patients (61%) (CYVADIC, n=12, 86%; cyclophosphamide 500 mg/m2 and doxorubicin 50 mg/m2 on day 1, vincristine sulfate 1.5 mg/m2 on days 1 and 5, and dacarbazine 250 mg/m2 on days 1 through 5 for three to five monthly cycles); IAP, n=1, 7%; doxorubicin 50 mg/m2 and cisplatin 50 mg/m2 on day 1 and ifosfamide 1000 mg/m2 on days 1 through 5 for three monthly cycles; and weekly TC, n=1, 7%; paclitaxel 60 mg/m2 and carboplatin AUC 1.7 on day 1 for 16 weekly cycles). The benign controls were 20 specimens of uterine leiomyomas that were removed surgically in the same period. At least 2 sections were isolated from each tumor and a tumor with at least one stained section was judged to be positive. The study was approved by the institutional review board and informed consent was obtained from all patients.

Immunohistochemistry and histological examination

Formalin-fixed and-paraffin-embedded tissue blocks were cut into 4-μm sections for immunohistochemistry and hematoxylin and eosin staining.

The presence of a malignant mesenchymal tumors was examined based on positive staining for SMA (Sigma 1A4, Sigma Chemical Co., St. Louis, MO), vimentin (clone V9, DakoCytomation, Glostrup, Denmark), desmin (clone D33, DakoCytomation, Glostrup, Denmark) and MIB-1 (Ki-67/clonal MIB1, DakoCytomation, Glostrup, Denmark), and negative staining for CD34 (QBEnd/10, Novocastra, Newcastle, UK) in an initial histological examination.

GLUT-1 expression was evaluated immunohistochemically using rabbit polyclonal anti-human GLUT-1 antibody (DAKO, Carpinteria, CA, USA) at a dilution of 1:50. HK-II expression was evaluated with a polyclonal rabbit anti-HK-II antibody (Chemicon International, Inc., Temecula, CA) diluted at 1:500. The sections were washed and then incubated with anti-rabbit IgG conjugated to horseradish peroxidase-labeled-dextran polymer (EnVision Kit, DAKO) for 60 min at room temperature. The extent of GLUT-1 and HK-II expression was assessed semi-quantitatively according to the following scoring scheme: negative staining (–), weak to sporadic staining (1+), and strong staining (2+). In judging the staining level, erythrocytes and pancreatic tissue were used as positive controls for GLUT-1 and HK-II, respectively.

The histological grade of LMS was evaluated by two expert pathologists by counting of 2000 cells, with >20 mitosis events in 10 microscopic fields judged to be positive. These data were used to calculate the MIB-1 index for cell proliferation.

Statistical analysis

The relationship between immunohistochemical scores and MIB-1 index was determined by linear regression for the GLUT-1 and HK-II data. Survival curves related to immunoreactivity were constructed using the Kaplan-Meier method and assessed by log-rank test. $P<0.05$ was considered to indicate significance in all analyses.

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III. Results

Hematoxylin & eosin and immunohistochemical staining

Malignant mesenchymal tumors identified from positive staining for SMA, vimentin, desmin and MIB-1 and negative staining for CD34 were excluded from the study (Fig. 1).

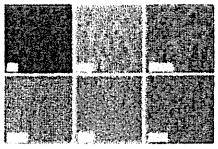


Fig. 1
Malignant mesenchymal tumors detected with hematoxylin-eosin (HE) staining, positive immunostaining for SMA, vimentin, desmin and MIB-1, and negative immunostaining for CD34. Bar=100 μm.

GLUT-1 and HKII expression, and MIB-1 index

Of the 23 cases of stage I LMS, 12 (52.2%) were GLUT-1-positive, including 2 2+ and 10 1+ cases; and 15 (65.2%) were HK-II-positive, including 1 2+ and 14 1+ cases (Table 1, Figs. 2, 3). The MIB-1 index was ≥5% in 10 cases and <5% in 13 (Fig. 4). Of the 20 benign controls, 2 (10%) were GLUT-1-positive, 4 (20%) were HK-II-positive, and all showed 1+ staining. MIB-1 was negative in all control specimens (Table 2).

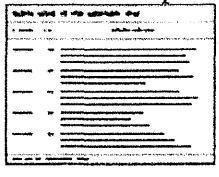


Table 1
Characteristics of patients with stage I uterine leiomyosarcoma

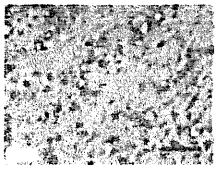


Fig. 2
Immunohistochemical staining showed strong GLUT-1 expression in sarcoma cell membranes. Bar=100 μm.

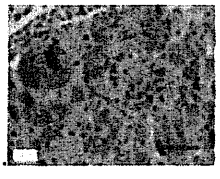
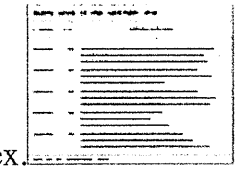


Fig. 3
Immunohistochemical staining was strongly positive for hexokinase (HK)-II in sarcoma cells. Bar=100 μm.

Fig. 4



Simple regression analysis of GLUT-1 expression and the MIB-1 index.

Table 2

Characteristics of patients with uterine leiomyoma**Relationships of GLUT-1 and HK-II expression with the MIB-1 index**

GLUT-1 expression levels showed a significant correlation with the MIB-1 index (R^2=0.403, p=0.0011, Fig. 4). In contrast, there was no correlation between HK-II expression and the MIB-1 index (p=0.703, Fig. 5).

Fig. 5

Simple regression analysis of HK-II expression and the MIB-1 index.**Survival curves based on GLUT-1 expression and HK-II expression**

The Kaplan-Meier estimates of overall survival (OS) at 10 years for LMS were 90.9% in the GLUT-1-negative group and 58.3% in the GLUT-1-positive group, showing a significant difference in prognosis (Fig. 6). The Kaplan-Meier estimates of OS at 10 years were 75.0% and 73.3% in the HK-II-negative and HK-II-positive groups, with no significant difference between these groups (Fig. 7).

Fig. 6

Survival curves for GLUT-1-positive and GLUT-1-negative cases of LMS.

Fig. 7

Survival curves of HK-II-positive and HK-II-negative cases of LMS.Go to:

IV. Discussion

Uterine LMS is likely to show recurrence and metastasis, even in the early stage, and effective treatment has not been established. In 1985, Omura *et al.* evaluated 48 cases of stage I and II uterine LMS in a randomized comparative study, and found recurrence rates of 44% in patients given adjuvant chemotherapy of 8 cycles of doxorubicin after resection and 61% in those who underwent observation only, with no significant difference between the groups [27]. A more recent randomized phase III trial of adjuvant pelvic radiotherapy versus observation for stage I and II uterine sarcomas (carcinosarcoma, leiomyosarcoma or endometrial stromal sarcoma) indicated that radiotherapy did not contribute to control of local metastasis or survival rate [32]. Hensley *et al.* conducted a prospective study in 23 cases (stage I: 15, II: 3, III: 1, and IV: 4) of high grade uterine LMS for a mean period of 49 months after complete resection, and found that progression free survival (PFS) at 2 years was 45% after treatment with gemcitabine 900 mg/m2 (on days 1 and 8 i.v.) plus docetaxel 75 mg/m2 (on day 8 i.v.) for 4 cycles at 3-week intervals. The PFS in stage I and II cases at 2–3 years was 59%, which suggested that adjuvant chemotherapy with gemcitabine plus docetaxel after complete resection may improve the prognosis of early stage LMS [13]. Several pilot studies of adjuvant therapies, including CYVADIC

(cyclophosphamide, vincristine, doxorubicin, and dacarbazine) therapy, ifosfamide single therapy, and API (doxorubicin, cisplatin and ifosfamide) plus radiotherapy have been conducted for early stage LMS [21, 26, 30], with 3- and 5-year survival rates ranging from 67% to 89% (one study with CYVADIC therapy had a 15-year survival rate of 69%).

There is currently no established surgical procedure or anticancer treatment for uterine sarcoma. This may be because of the relatively small number of cases of uterine sarcoma and because the disease is often not diagnosed before surgery. Cases 1 and 2 were young patients who underwent myomectomy and were diagnosed with uterine sarcoma in a postoperative pathologic examination. Consequently, these patients underwent hysterectomy in an abdominal reoperation. Several small-scale studies have indicated that CYVADIC chemotherapy improves prognosis after total hysterectomy and adnexectomy [12, 31, 41]. In our study, no gross residual tumor was found during lymph node dissection. Twelve patients (52%) were treated with CYVADIC chemotherapy and 9 (39%) did not receive this chemotherapy. Three patients died in each of these groups. Most previous studies and the current study were performed at single centers and with a limited number of patients. Therefore, multicenter randomized clinical trials are required to establish more reliable evidence of the efficacy of treatment.

In our previous investigation of different histological types (serous, mucous, endometrioid and clear cell) of epithelial ovarian cancer, we found that expression levels of GLUT-1 and HIF1α were correlated in the respective histological types. Expression of both proteins was especially high in serous adenocarcinoma, which is frequently found in epithelial ovary cancer, and clear cell adenocarcinoma, which is chemoresistant and associated with recurrence and metastasis. Histopathologically, these two tumors have fewer vascular vessels, but have papillary proliferation and a stratified structure, and cause extensive necrosis in progression. Therefore, hypoxia is induced as the cancer progresses, and this leads to strong expression of GLUT-1 and HIF-1α [16, 43].

Many studies have evaluated the relationship between the expression level of GLUT-1 and progression of epithelial and gynecologic cancers, with the general finding that strong GLUT-1 expression is associated with a poorer prognosis [1, 6, 11, 17, 19, 44, 45]. In a study of 67 patients with bone and soft tissue sarcoma or non-epithelial carcinoma (stage IA–IVB, 15 different histological types), Endo *et al.* found a correlation of prognosis with therapy including surgical resection and adjuvant chemotherapy ($p<0.0001$), tumor differentiation ($p=0.017$), necrotic grade ($p=0.04$), mitotic grade ($p=0.0198$), MIB-1 grade ($p=0.031$), and GLUT-1 expression ($p=0.029$) in univariate analysis of 3-year survival; but found that metastasis ($p=0.031$) was the only significant prognostic factor in multivariate analysis of overall survival. However, Kaplan-Meier estimates of overall survival at 5 years were <40% in the GLUT-1-positive group and 90% in the GLUT-1-negative group, showing a significant difference in prognosis [8].

In the present study, all the cases of non-epithelial carcinoma were in clinical stage I, involved uterine LMS with a single histological type, and were completely resectable in surgery. Thus, there was less variation in the subjects in this study in comparison with Endo *et al.*, which included cases of different histological types and progression. The 5-year OS of all LMS patients was approximately 74%, and the MIB-1 index and expression level of GLUT-1 had a significant positive correlation. The 5-year survival rates were 90.9% and 58.3% in GLUT-1-negative and GLUT-1-positive cases, respectively. Thus, GLUT-1 expression was significantly correlated with prognosis in uterine LSM, as found in previous studies of malignant epithelial tumors. In contrast, there was no relationship of HK-II expression with the MIB-1 index or prognosis, and no significant difference in survival between HK-II-positive and HK-II-negative cases.

Previous studies have shown that expression of GLUT-1 and HK-II in epithelial cancer cells, including breast, esophageal, and lung cancer cells, plays a pivotal role in glucose metabolism and that the expression levels of GLUT-1 and HK-II are correlated with malignancy [5, 14, 17, 23, 28, 38]. The subjects of the current study were 23 patients with LMS associated with non-epithelial malignancy and the prognosis correlated with the presence or absence of expression of GLUT-1 in malignant cells, but not

with expression of HK-II. Therefore, it is possible that the occurrence of malignant cells depends on glucose metabolism, glucose enzyme activity and phosphorylation, but not on epithelial cell malignancy.

The recent development of [18F]-fluorodeoxyglucose positron emission tomography (FDG-PET) allows imaging based on the difference in glucose metabolism between malignant and normal cells. Thus, FDG-PET is effective for detection of early stage malignant tumors, and has high sensitivity for detection of recurrence and metastasis in malignant gynecological epithelial tumors [3, 4, 20, 35]. In the first study of non-epithelial bone and soft tissue sarcomas using FDG-PET, Tateishi *et al.* showed an association between higher GLUT-1 expression and a higher standardized uptake value (SUV) of [18F]fluorodeoxyglucose, thereby suggesting the efficacy of FDG-PET diagnostic imaging for non-epithelial tumors, as well as MIB-1 grade, mitotic grade, and tumor differentiation [36].

We have also evaluated FDG-PET for early detection of recurrence and metastasis of advanced ovary cancer after treatment. The rates of detection of intraperitoneal and retroperitoneal metastasis by FDG-PET were 93.9% and 92.9%, respectively, and metastasis was detected in 14 (50%) of 28 metastatic lymph nodes of normal size. FDG-PET detected recurrence in 87.5% of CA125-positive patients with no symptoms and negative results in conventional CT and ultrasonography [24]. The efficacy of follow-up FDG-PET was evaluated in patients with uterine LMS, and early-stage minimal recurrent lesions were detected in patients in whom conventional CT and ultrasonography did not show intraperitoneal recurrence. Two of 5 patients underwent reoperation for a recurrent tumor and survived for one year or more after surgery.

Benign non-epithelial tumors such as uterine leiomyomas are rarely positive in PET, but such cases should be followed up carefully because some may be false-positives [39]. However, FDG-PET is effective for detection of early-stage intraperitoneal recurrence that may be overlooked in conventional diagnostic imaging [25]. The clinicopathological results reported here show that malignant non-epithelial tumors have high glucose metabolic activity and high GLUT-1 expression, and these findings support the use of FDG-PET for detection of malignant lesions.

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V. Acknowledgments

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VI. References

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