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Impact of Tumor Angiogenesis in Peritoneal Mesothelioma After Radical Cytoreduction and Hyperthermic Intraperitoneal Chemotherapy

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Abstract Peritoneal mesothelioma is one of the peritoneal surface malignancies where long-term survival is a reality after cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC). Tumor angiogenesis has been shown to be of prognostic significance on survival in mesothelioma. We investigated the impact of survival of patients with peritoneal mesothelioma following CRS and HIPEC to determine the impact of tumor angiogenesis on survival after this radical surgical treatment. Paraffin sections of 23 patients who were treated with CRS and HIPEC were retrieved for immunohistochemical analysis. The immunostaining was performed using monoclonal mouse anti-human antibodies (VEGF-C and CD31) on an autostainer (Autostainer Plus; Dako, Inc.). The intensity of the stains were quantified using the Image-Pro Plus (IPP) 4.5 (Media Cybernetics, Silver Spring, MD). VEGF expression and microvessel density (MVD) using CD31 staining were studied. The median survival was 94 months with a 3-year survival rate of 51%. There was no impact on patient's age, sex, peritoneal cancer index, tumor histopathology and survival outcomes between patients with low or high MVD and VEGF expression. After CRS and HIPEC, our results demonstrate that the prognostic significance of tumor angiogenesis is negated, highlighting the potential importance of other co-contributory mechanisms in mesotheliomagenesis and undergoing radial treatment.

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Introduction

Peritoneal mesothelioma is a malignancy that arises from mesothelial cells found within the serosal lining of the peritoneum and comprising of one-third of all mesothelioma [1]. Mesothelioma is a rapidly progressing, asbestos-related tumor. This tumor has emerged from being a rarity and is now showing an increasing incidence worldwide following the enormous historical production and use of asbestos in the various construction materials and commercial products that have resulted in millions of people being exposed [2]. Basic and translational oncology research has identified the putative role of tumor angiogenesis as an important prognostic factor on survival in mesothelioma [3–10]. In these studies, the association of the expression of tumor angiogenesis is based on vascular endothelial growth factor (VEGF) expression or microvessel density and patient survival outcomes is based on palliative treatments that include systemic chemotherapy, decortications, pleurodesis or biopsy only, with few patients undergoing radical surgery [3, 5–8].

Angiogenesis is a fundamental event in the process of tumor growth and metastasis [11]. The underlying molecular events mediating this have been identified to occur through angiogenic cytokines of which vascular endothelial growth factor (VEGF). The VEGF family of molecules comprises of multiple ligands and receptors whose signalling process and binding leads to the promotion of endothelial cell proliferation, migration and survival from

pre-existing vasculature and lymphatic vessel formation [12, 13]. From the aforementioned studies, it appears that the extent of tumor angiogenesis predicts for a poorer survival outcome.

Treatment of this disease have evolved from systemic chemotherapy only, where median survival is less than 12 months [14, 15] to a radical surgical approach of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for peritoneal mesothelioma where median survival of up to 34 to 92 months may be achieved [16], and extrapleural pneumonectomy with intracavitary intraoperative hyperthermic chemotherapy for pleural mesothelioma, where median survival is 17 months [17]. Given the augmented survival outcome of patients following radical treatments where long-term survival is achieved in patients undergoing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy, we seek to determine the prognostic significance of tumor angiogenesis after this radical treatment.

Patients and Methods

Patients

Twenty-three patients with a clinical and histopathological diagnosis of peritoneal mesothelioma underwent CRS and HIPEC by a single surgeon team (D.L.M) according to Sugarbaker's protocol [18]. HIPEC was performed for 90 min using an open abdomen technique with cisplatin (50 mg/m^2) and doxorubicin (15 mg/m^2) in 3 l of 1.5% dextrose peritoneal dialysis solution at 42°C. A signed informed consent was obtained from all patients for their clinical information to be used in research and to donate the left-over tissue after the completion of histological diagnosis.

Clinical Data

All clinical data were obtained prospectively and stored. The clinical variables recorded include; age at time of surgery, sex, peritoneal cancer index (PCI), completeness of cytoreduction, histological subtype, and overall survival.

Immunohistochemistry

Tumor specimens from patients were harvested and preserved as paraffin-embedded tissue. Paraffin blocks were sectioned as 4 to 5 μm sections and mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX) by S.E.A.L.S. Pathology, St George Hospital. These sections were used for immunohistochemistry staining for VEGF protein and CD31 antigen. One section from each sample was also stained with hematoxylin and eosin (H&E) to facilitate histological assessment. The staining process was performed according to a standard protocol. Briefly, sections were baked at 60°C, cooled and deparaffinized in xylene, 100% ethanol, 95% ethanol, 70% ethanol, and de-ionized water. The slides were treated with EDTA retrieval solution (pH 9.0) or citrate-based retrieval solution (pH 6.0) for 20 min at 95°C, and blocked with 0.3% hydrogen peroxide before the application of primary antibodies; monoclonal mouse anti-human VEGF (C-1) Antibody sc-7269 (Santa Cruz Biotechnology, Inc) and monoclonal mouse anti-human CD31, Endothelial Cell (Dako Aust. Pty Ltd, Botany, Australia). Immunostaining was performed on an autostainer (Autostainer Plus; Dako, Inc.). This involved incubating with monoclonal mouse anti-human antibodies (VEGF and CD31 antibodies) for 1 hour at room temperature. The sections were further incubated with goat anti-mouse HRP-Streptavidin immunoglobulin (Abcam) for 30 min at room temperature. The slides were developed with diaminobenzidine (DAB; Dako) for 10 min and counterstained with hematoxylin. Two types of negative controls, substituting the matched mouse IgG isotype and goat nonimmune IgG in the staining protocol, were used.

Following immunostaining, the slides were first analyzed manually. Any cytoplasmic staining for VEGF and membranous staining for CD31 were considered positive respectively. The immunostaining results were evaluated by defining a threshold of positive staining for all sections before automated processing. Briefly, the threshold of positive signal was defined for each antibody for all the sections following different treatments. Colour signal above the threshold for each antibody defined was deemed to be positive, whereas any signal below the threshold was regarded as negative. The intensity was averaged from ten fields of view. This was performed using Image-Pro Plus (IPP) 4.5 (Media Cybernetics, Silver Spring, MD). All images analyzed with IPP 4.5 were counter-checked against the H&E slide to ensure accurate histomorphometry.

To assess the microvessel density (MVD), the immunostained CD31 section is scanned at low magnification (40X) the area of clear-cut cancer tissue with the greatest number of distinctly highlighted microvessels was selected. MVD was then determined, by counting all vessels at a total magnification of 200 in an examination area of 0.25 mm^2. MVD was then determined by counting all vessels at a total magnification of 200X using an eye-piece screen with an edge length of 10 mm/100 and an examination area of 0.25 mm^2. Determination of the staining reaction for CD31 antigen was strictly confined to the area of highest microvessel density within or immediately adjacent to each tumor. In the following,

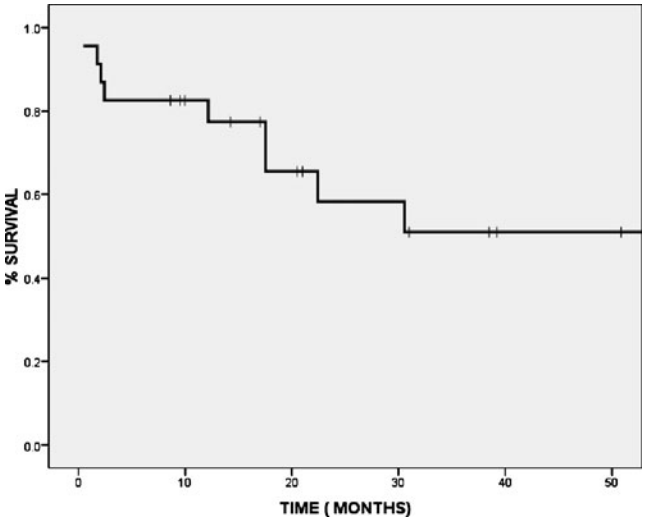

Fig. 1 Overall median survival of 23 patients undergoing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for peritoneal mesothelioma

MVD values are given as the value for one field of 0.25 mm^2. Any endothelial cell was regarded as a single countable microvessel, regardless of whether a lumen was visible or not. Unstained lumina were considered artefacts even if they contained blood or tumor cells. MVD was determined by one investigator (PY) who was blinded to the clinical outcomes of the patients.

Statistical Analysis

The data were analyzed using SPSS® for Windows version 16.0 (SPSS, Munich, Germany). The patient characteristics and immunostaining were compared using unpaired *t*-test and Fischer's exact test. The Kaplan-Meier method was used to analyze survival. The log-rank test was used to compare differences in survival. Survival was measured from the time of surgery. No patient was lost from follow-up.

Results

Twenty-three patients were treated with CRS and HIPEC. There were 15 men and 8 women. The median age at the time of surgery was 50.5 (range; 32.7 to 71.5) years. The extent of tumor dissemination assessed according to the peritoneal cancer index [19] was 16 (range; 8 to 39). Fifteen patients (65%) had a complete cytoreduction (CC0), seven patients (31%) had a near complete cytoreduction (CC1) and one patient (4%) had an incomplete cytoreduction (CC2). Following histopathological assessment, three patients were observed to have sarcomatoid tumors and 19 patients had epithelioid tumors and one patient had multi-cystic type tumor.

Survival Outcomes

The median follow-up period was 18 (range; 1 to 94) months. The median survival of 23 patients who underwent CRS and HIPEC was 94 months with a 1-, 2- and 3-year survival rates of 83%, 58% and 51% (Fig. 1). Patients who underwent a complete cytoreduction (CCR0) had a longer overall survival compared to patients who had an incomplete cytoreduction (CCR1/2/3) (94 vs. 17 months; p=0.008) (Fig. 2).

Expression of Immunostaining Markers on Survival Outcomes

The mean (standard deviation) and median (range) of MVD and VEGF staining were 11.7 (5.1) and 11 (range, 5 to 24); and 3.6 (3.3) and 3.0 (0.1 to 12.7) respectively. Low and high MVD and VEGF expression was based on a classification of MVD ≤10 or MVD >10 and VEGF ≤3 or VEGF >3. Between groups, there were no differences in patient's age, sex, peritoneal cancer index and tumor histopathology on MVD and VEGF expression (Table 1). Log-rank analysis to compare survival outcomes following CRS and HIPEC showed no effect of MVD (p=0.62) and VEGF expression (p=0.68) on overall survival (Figs. 3 and 4).

Discussion

Peritoneal mesothelioma remains a poorly understood malignancy for which the molecular biology of tumorigenesis remains unknown. Its aetiology has been related to a few

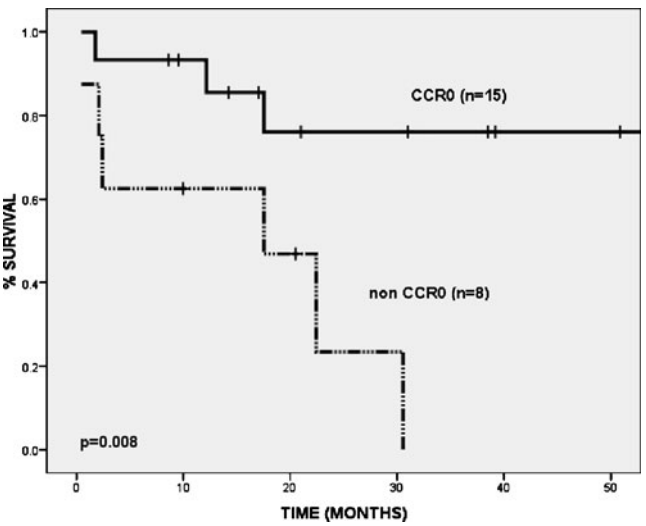

Fig. 2 Log-rank comparison of completeness of cytoreduction (CCR) on survival after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy where CCR0 is compared against CCR1/2/3

Table 1 Group comparison of 23 patient with peritoneal mesothelioma treated with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy and measures of tumor angiogenesis (VEGF and microvessel density)

	Microvessel density		*p* value	VEGF		*p* value
Mean (s.d.)	11.7 (5.1)			3.6 (3.4)		
	≤10	>10		≤3	>3	
Age (years)			0.10			1.00
≤51	4	10		7	7	
>51	6	3		4	5	
Sex			0.38			1.00
Male	8	7		7	8	
Female	2	6		4	4	
Peritoneal cancer index			0.68			0.41
≤15	6	5		6	5	
>15	5	7		4	8	
Tumor histology			1.00			0.59
Epithelioid	9	11		9	11	
Non-epithelioid	1	2		2	1	

hypothesis where preliminary investigations have led to derived some results that may substantiate these hypotheses.

Firstly, some studies have shown a relationship between chronic inflammatory process following irritation of asbestos fibres inducing malignant changes [20, 21]. Secondly, the presences of asbestos fibre have been shown to interfere with the process of mitosis leading to chromosomal instability, aneuploidy and other forms of chromosomal damage [22–24]. Lastly, asbestos induces phosphorylation of the mitogen-activated protein kinases and extracellular signal-regulated kinases 1 and 2 and elevate expression of early response proto-oncogenes (*FOS* or *JUN* or activator protein 1 family members) in mesothelial cells thereby causing persistent kinase mediated signaling which leads to persistent mesothelial cell proliferation [25, 26]. It has also been demonstrated that the Simian vacuolating virus 40 (SV 40) may contribute through an uncertain oncogenic effect on tumor suppressor genes to cause mesothelioma [27].

The importances of tumor angiogenesis on cancer proliferation and metastasis have been an enormous field of interest in the last two decades. Numerous studies have investigated the role of tumor angiogenesis through translational studies to determine its impact on survival and results have shown an association between a highly angiogenic tumor with a poorer survival [3–9]. However, no studies have demonstrated the independent effect of tumor angiogenesis on survival, indicating that this may be a co-contributory effect the aggressiveness of the tumor. These studies identified have also primarily obtained tumor

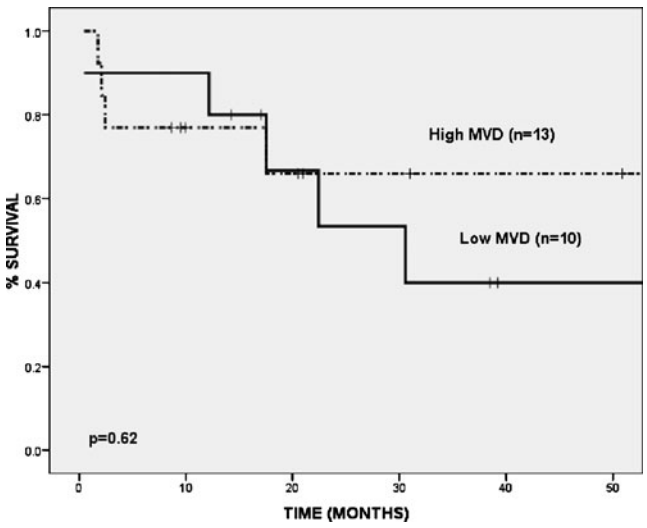

Fig. 3 Log-rank comparison of microvessel density (MVD) on survival after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy where low ≤10 and high >10

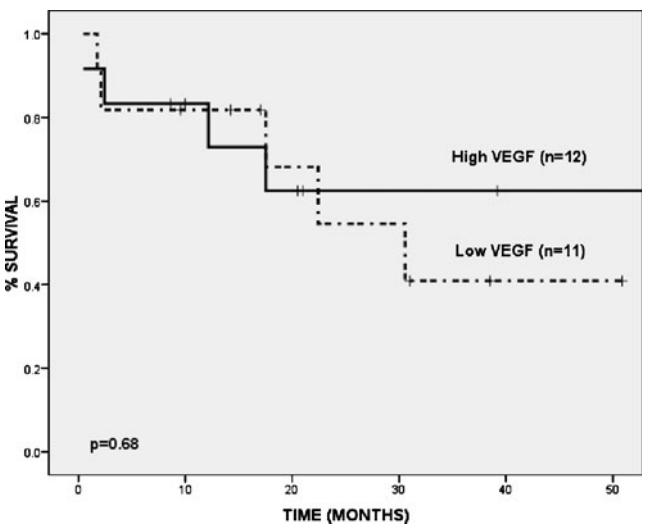

Fig. 4 Log-rank comparison of vascular endothelial growth factor (VEGF) expression on survival after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy where low ≤3 and high >3

specimens for correlation studies from palliative operations that include decortications, pleurodesis or biopsies and subsequent systemic treatments. These treatments may in this current era be regarded as palliative in the presence other radical procedures such as extrapleural pneumonectomy with intracavitatory intraoperative hyperthermic chemotherapy for pleural mesothelioma or cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for peritoneal mesothelioma.

In our study that adopted a radical procedure for treating patients with peritoneal mesothelioma, the median survival of patients after treatment was 94 months with a 3-year survival of 51%. This result corroborates enormous survival benefits that are demonstrated in a systematic review by Yan et al. [16], who studied the outcomes of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy of 240 patients reported in seven prospective observational studies from six tertiary institutions where a median survival ranging from 34 to 92 months and a 3- and 5-year survival of 43% to 65% and 29% to 59% was observed respectively. These studies of radical surgical cytoreduction and hyperthermic loco-regional chemotherapy administration are recognised to be improve overall survival compared to historical controls treated with palliative surgery and systemic chemotherapy. As such, this prolonged survival following treatment might have resulted in the loss of the prognostic significance of tumor angiogenesis on survival in our study where the expression of VEGF and microvessel density did not influence survival outcomes. In addition, the effect of radically remove the diseased peritoneum may have removed the microenvironment for which these malignant mesothelial cells arise from hence preventing its spread.

In a study by Aoe et al. [28] who studied the expression of VEGF of 37 tumor samples and correlated the expression levels with clinicopathological variables and overall survival, these authors found no statistically significant association between VEGF expression and gender, age, clinical stage and survival. Further, a study by Ohta et al., showed no difference in VEGF mRNA in tissue samples between patients with early and advance stage disease [10]. In another study by Demirag et al. [6] who reported the prognostic significance of VEGF, tumor necrosis and mitotic activity index in malignant pleural mesothelioma, their expression of VEGF with tumor stage (p=0.046) and only short survival (p=0.0002). Edwards et al. [5] who reported that angiogenesis was associated with poor survival studied 93 cases and assessed the microvessel density and its impact on survival. In their study, the overall median survival was only 5 months and a significant correlation of microvessel density (p=0.01) and survival was demonstrated. In another similar study by Ohta et al. [10], it was shown that patients with a high vessel density survived 11 months compared to patients with a low vessel density who survived 17 months (p=0.03).

In conclusion, the findings of our study showed that in long-term survivors of patients with peritoneal mesothelioma who underwent radical cytoreduction and hyperthermic intraperitoneal chemotherapy, there is no influence of tumor angiogenesis on survival compared to its influence on patients with a short-term survival from palliative treatments, hence suggesting that the prognosis would be influenced by other more importance mechanisms in mesotheliomagenesis and radical surgical treatments.

Conflict of Interest None.

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