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Expression of Chemokine Receptor CXCR4 in Primary and Metastatic Neuroblastoma

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ABSTRACT

Neuroblastoma is the most common neuroendocrine childhood tumour having a potential to metastasize to lymph nodes, bone marrow, bones and liver. All of these metastatic sites were shown to express a chemokine receptor; CXCR4. CXCR4 has been involved in the directional homing of tumour cells to metastatic sites rich in stromal cell-derived factor-1 (SDF1). The Aim of this study was to investigate expression of CXCR4 in both the primary and metastatic clinical samples of neuroblastoma. A total of 31 archived tissue specimens of neuroblastoma were subjected to immunohistochemical staining using a monoclonal antibody specific for CXCR4. CXCR4 expression grades were compared with demographic and clinical characteristics for each patient. Results showed that CXCR4 was significantly expressed in primary and metastatic neuroblastoma. There was high CXCR4 expression in tumors with advanced stage of disease which correlated with increased metastatic potential to bone marrow. High CXCR4 expression was shown to connote unfavourable disease prognosis especially in patients with advanced stage of neuroblastoma. Consequently, aberrant CXCR4 expression was significantly associated with unfavorable disease prognosis and high metastatic spread to bone marrow in children with primary and metastatic neuroblastoma.

Key words: Cancer, Chemokine, CXCR4 , Immunohistochemistry, Neuroblastoma

INTRODUCTION

Neuroblastoma is a childhood cancer where uncontrolled metastasis is the major cause of death. It is an embryonic, extracranial solid tumour and is being the second most common paediatric tumour accounting for 10% of paediatric tumours and for 15% of childhood fatalities[1]. It is derived from neural crest cells of the sympathetic nervous system, with most primary tumours arising in the abdomen, where the adrenal gland is the most common site. Other primary locations include the neck, chest and pelvis[2]. Common metastatic sites are the lymph nodes, bone marrow, bones and liver[3].

Chemotherapy plays a crucial role and remains the predominant treatment in the management of intermediate and high-risk neuroblastoma. Several chemotherapeutic agents have been utilised individually or in combinations for the treatment of neuroblastoma. However these are still poor and it is estimated that more than half of the children with advanced-stage disease will relapse because of drug-resistant residual disease[4]. Furthermore, long-term toxicity of chemotherapy remains an essential issue[5]. Novel therapeutic approaches are therefore needed for the treatment of neuroblastoma.

One of the interesting molecules that received a considerable attention in the last decade is Chemokine receptor-4 (CXCR4). It is small chemotactic protein that belongs to large superfamily of G-protein-coupled receptors. It plays an important role in regulating several biological processes not only in organogenesis, hematopoiesis, and immunity but also in tumour progression and dissemination[6]. Several reports demonstrated the over-expression of CXCR4 in different types of human cancers including kidney, lung, brain, prostate, breast, pancreas, ovarian, and melanomas. Further studies pointed to the role of CXCR4 in mediating physiological and pathological process contributing to tumour growth, angiogenesis and metastasis[6, 7]. Recently, CXCR4 and its ligand, stromal cell-derived factor-1 (SDF-1), was found to be important in mediating the homing of neuroblastoma cells to bone marrow leading to preferential formation of bone marrow metastasis. Neuroblastoma cells preferentially metastasize to lymph nodes, bone marrow, bones and liver, all of which are sites having high levels of SDF-1 [8]**.** Moreover, CXCR4/ SDF-1 signalling axis showed to enhance the primary tumour and metastatic growth of neuroblastoma cells while no effect was shown on cell invasion[9].

On this basis, CXCR4 represents a unique and novel therapeutic opportunity to develop a new strategy for the treatment of neuroblastoma. One potential approach would be to modulate the expression of CXCR4 in neuroblastoma cells which are involved in the process of tumour progression and dissemination. Whilst there have been some studies looking for CXCR4 role in tumours, none have attempted to investigate CXCR4 expression in both primary and metastatic tumours. Therefore, the aim of this study was to investigate CXCR4 expression in both primary and metastatic tumours and to correlate it with degree of clinical metastasis to these tissues.

MATERIALS AND METHODS

Tissue specimens

Archived Tissue specimens of neuroblastoma were surgically obtained from 31 patients who previously underwent surgery in the Department of Surgery, King Hussin Medical Hospital, Royal Medical Services. Seventeen samples were metastatic samples of bone marrow and lung whereas other samples were primary tumours located in abdomen, spine, paraspine, sacrum, brain and pharynx. All personal data for samples were kept anonymous. The study was ethically approved by the Ethics Committee, Faculty of Medicine, Mutah University.

Immunohistochemistry

Archived, five µm-thick formalin-fixed, and paraffin-embedded (FFPE) tissue sections were deparaffinised and rehydrated by transferring them through xylene and serial dilutions of alcohol. To block endogenous peroxidase activity, the sections were treated for 5 minutes with 3% hydrogen peroxide.Antigen retrieval was performed by microwaving the slides at 600W for 20 minutes in citrate buffer (10 mM citrate buffer, pH 6.0).Prior to antibody incubation, the non-specific binding of the antibodies was blocked by incubating the sections for 20 minutes at room temperature with 1.5% normal goat serum (Vector Laboratories Ltd, Peterborough, UK) . Sections were then incubated with rabbit monoclonal anti-CXCR4 (clone: UMB2, Abcam, UK) at concentration of (20 µg/mL) for overnight at 4cº. Following primary antibody treatment, each section was incubated with goat anti-rabbit peroxidase-conjugated secondary antibody (Vector Laboratories Ltd, Peterborough, UK) (7.5 µg/mL) for 30 minutes at room temperature. Immunoreactivity was visualised by incubating sections with 3,3-diaminobenzidine tetrahydrochloridechromogen (DAB) (Vector Laboratories Ltd, Peterborough, UK) solution for 3-5 minutes. Following colour development, sections were then counterstained in Harris's haematoxylin

solution mounted with coverslips using DPX medium.

The resulting slides were viewed and analyzed by using a Leica DMRB microscope (Leica DMRB, Wetzlar, Germany) with the images digitally captured and processed using a Leica MPS52 camera (Q Imaging, Germany) and the AcQuis imaging capture system (Synoptics, Cambridge, UK) respectively.

Scoring

The scoring system used to analyze immunohistochemical labeling of neuroblastoma clinical samples was based on previously published studies[10,11]. For CXCR4 expression, cells were considered positive if they demonstrated clear cytoplasmic and/or nuclear immunolabeling. The scoring results were calculated as percentage of cells showing positive expression as opposed to strength of staining in an individual cell. The scoring results of each immunolabeling of specific cell types were presented as: none (0), weak (1), moderate (2) and high (3). The score 'none' indicated an absence of expression. Sections were allocated a score of 'weak' when less than 33% of cells had expression. A score of 'medium' was applied to cells which had expression on 33% to 66% of the section whilst the score 'high' represented sections which had expression on more than 67% of the cells.

Statistical analysis

The data was appropriately coded and subjected to analysis using the Nonparametric Spearman's rank order correlation coefficients using SPSS software (version 16.0, Chicago, IL)

RESULTS

Clinical and Demographic Characteristics

All the demographic and clinical data in about 31 patients of neuroblastoma are presented in table 1. There was a male predominance (76.7%) while female constituted only 32.3% of the patients. The majority of the primary tumor sites were located in the abdomen (61%) where the most common site was in the adrenal glands. The most common sites of metastasis were in lymph nodes (77%) and in bone marrow (71%). Other metastatic sites include bone and lung. There were twenty two patients have metastasis for both lymph node and bone marrow. There was one patient with primary tumor in the brain which had no evidence of distant metastasis. In terms of disease staging, 42% of patients were clinically presented with advanced stage of disease (3 and 4), where as 58% of patients were at low stage of disease (1,2 and 4s). Make the sentence like this: According to International Neuroblastoma Pathology Classification (INPC), 77% of these patients had unfavourable prognosis.

Table 1: Patient Characteristics. INSS: International Neuroblastoma Staging System, INPC: International Neuroblastoma Pathology Classification

Characteristics	Number/ Percentage (%)
Age	
Mean	5.9
Gender	
Male	21(76.7)
Female	10 (32.3)
Site of primary tumor	
Abdomen	19 (61)
Reteropharyngeal	1 (3)
Spine	3 (10)
Paraspine	3(10)
Sacrum	1(3)
Thorax	3(10)
Brain	1(3)
Sites of metastases	
Bone	1(3)
Bone Marrow	22 (71)
Lymph node	24 (77)
Pulmonary	1(3)
INSS stage	
1	5 (17)
2	2 (6)
3	4 (13)
4	9 (29)
4S	11 (35)
INPC classification	
Favorable	7 (23)
Unfavorable	24 (77)
CXCR4 expression	
0	6%
1	19%
2	39%
3	36%

CXCR4 Expression in primary and metastatic neuroblastoma

CXCR4 was expressed in 84% of both primary and metastatic neuroblastoma clinical specimens. The staining was predominantly localised to the nucleus of tumour cells without any significant staining noted in the peripheral or cytoplasm (Figure.1). The immunostaining was uniform across all sections with varying degree of staining intensity. Thirty six percent of the neuroblastoma samples showed high CXCR4 expression with more than 66% of the cells expressing CXCR4. Twelve tumors (39%) showed moderate CXCR4 expression ranging between 33% to 66% of cells expressing CXCR4. There were six tumours (19%) showed weak CXCR4 expression with less than 33% of cells expressing CXCR4. Only two tumours (6%) had no staining at all. CXCR4 expression was more predominantly found in poorly differentiated cells than in well differentiated cells. Moreover, there was no significant difference in mean CXCR4 expression between the neuroblastoma primary tumours and metastatic ones.

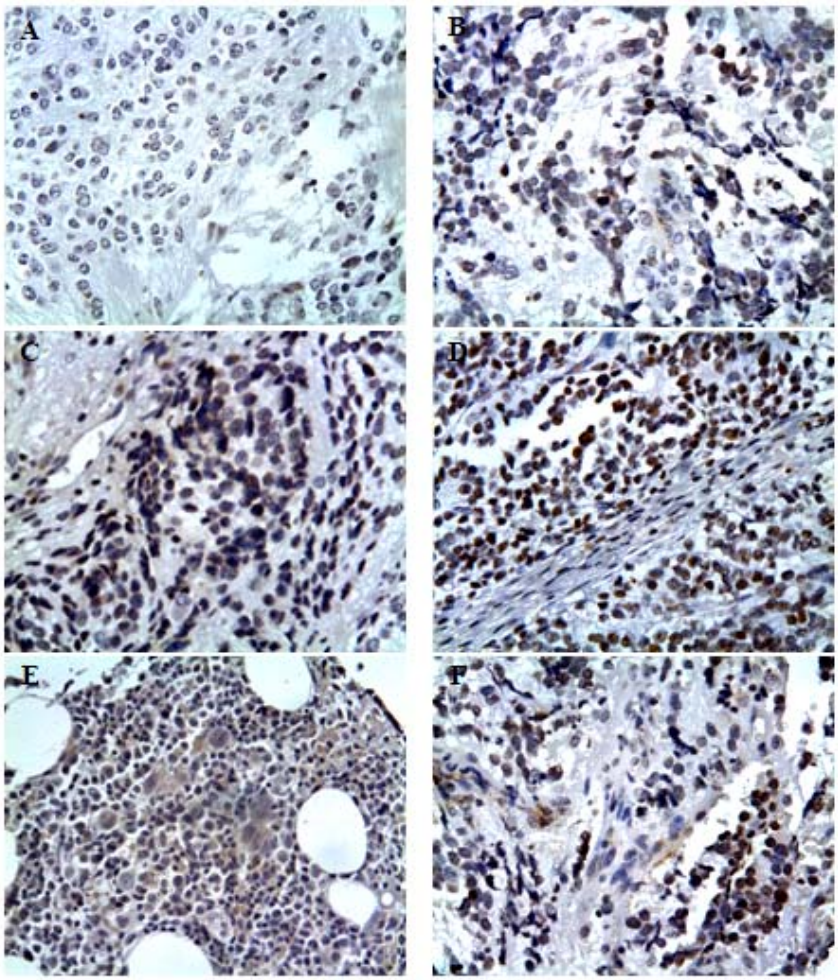

Fig. 1: CXCR4 expression in neuroblastoma primary and metastatic tumors. Tumors were classified on the basis of percentage of cells showing positive staining. (A) Grade 0, indicated an absence of expression. (B) Grade 1 indicated less than 33% of cells had expression. (C) Grade 2 applied to cells having expression on 33% to 66% of the tumor. (D) Grade 3 represented tumors having expression on more than 66% of the cells.(E) Expression of CXCR4 in bone marrow. (F) Expression of CXCR4 in pulmonary sample

CXCR4 expression is correlated with clinical stage, site of metastasis and prognosis

Clinical staging of neuroblastoma disease describes the extent of primary tumor growth and how far the disease is spread to regional lymph node and metastasizes to distant sites such as bone and bone marrow. CXCR4 expression was compared with International Neuroblastoma Staging System (INSS) stages of disease. There was a strong association between all grades of CXCR4 expression and clinical stages ($P \leq 0.05$). CXCR4 expression was found to be highly expressed in patients with advanced stage of disease (stage 3 and 4). Low levels CXCR4 expression was found in patients with low stage neuroblastoma (stage 1, 2 and 4s) (Figure. 2).

As the CXCR4 plays a role in mediating the homing of neuroblastoma cells to bone marrow, CXCR4 expression was also compared with the metastatic spread pattern in neuroblastoma disease. There was a significant correlation ($P \leq 0.05$) between increased CXCR4 expression and metastatic spread to lymph node and bone marrow. Correlations with other metastatic sites were not possible because of limited size of metastatic samples.

Neuroblastoma tumour histology has an important impact on prognosis of disease. International Neuroblastoma Pathology Classification (INPC) evaluates tumour specimens and classifies them to favourable and unfavourable prognoses on the basis of histological parameters and patient age. In this regard, the CXCR4 expression was also compared with prognosis of disease. There was a significant correlation ($P \leq 0.05$) between CXCR4 expression and prognosis of patients. Increased CXCR4 expression indicated unfavourable or poor disease prognosis especially in patients with advanced stage of neuroblastoma. On other hand, low levels of CXCR4 expression connoted favourable disease prognosis majorly in patients with low stage of neuroblastoma. Regarding the patient age and gender, there was no significant difference in the mean age and gender across all grades of CXCR4 expression.

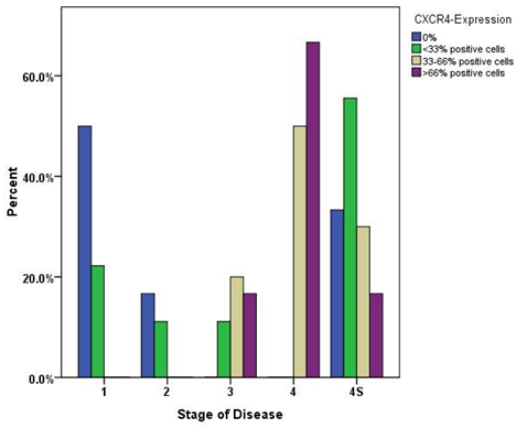

Fig. 2: Grade of CXCR4 expression in primary and metastatic neuroblastoma tumors versus clinical stage at presentation

DISCUSSION

Whenever metastatic disease occurs the disseminating neuroblastoma cells from the primary mass remain the main reason for increasing neuroblastoma morbidity and mortality. This is due to uncontrolled metastasis that restricts the normal organ function and therefore causes death. Treatment of metastatic disease remains difficult and represents a major challenge for health care systems. Once cancer has disseminated from the primary tumor, chemotherapy is the most widely used treatment modality as localized therapies (surgery and radiation) are often no longer effective. Despite major advances in the efficacy of chemotherapeutic agents many cancers are difficult to treat and remain incurable because of uncontrolled metastasis. Moreover, the lack of selectivity and resistance to chemotherapeutic agents remain an important issue[5,12].

Research has begun to focus on alternative targets for therapy including angiogenesis, invasion, migration and metastasis. The success of therapeutics in these areas has yet to be realized, with toxicities being observed. With this in mind, the focus of the research described in this study is CXCR4, which has an important role in the tumor dissemination process. The growing evidence shows that CXCR4/SDF1axis functions in homing of tumor cells to the site of metastasis[6, 7]. It is believed that CXCR4 expressing tumor cells undergo directional trafficking towards rich SDF1 organs such as lymph node, liver, lung and bone.

In SDF1- dependent manner, several tumors expressing CXCR4 such as neuroblastoma, breast and prostate cancers metastasize to the bones through the blood stream. Supporting this hypothesis, there was a reduction in the metastatic potential upon abrogation of CXCR4/SDF1axis in several animal models of cancers[7,13].

It is found that the homing and adhesion of neuroblastoma cells was mediated by CXCR4 expression. The trafficking of neuroblastoma cells to bone marrow was possibly mediated by a similar mechanism to homing of hematopoietic stem cells. CXCR4 was expressed in eight characterized neuroblastoma cell lines and that expression was found to a general characteristic of neuroblastoma cells. Moreover, the constitutive expression of SDF1 controlled the levels of CXCR4 expression in neuroblastoma cells. In other word, the presence of SDF1in neuroblastoma tumor microenvironment induced CXCR4 down-regulation and removal of ligand enhanced recovery of CXCR4 receptor. Moreover, neuroblastoma cells were showed to preferentially migrate and adhere to SDF1 rich bone marrow stromal cells[8].

Additional work on the role of CXCR4 in neuroblastoma tumor metastasis was performed by Zhang *et al*[14]. Consistent with other studies, CXCR4 was expressed in different neuroblastoma cells lines and its expression was found to be dynamically regulated and dependent on tumor microenvironment. Furthermore, over-expression of CXCR4 in neuroblastoma cells enhanced cell migration and increased metastatic capacity of these cells to bone marrow metastasis. Other evidence on the contribution of CXCR4/SDF1 axis in the growth of neuroblastoma metastasis came from Meier *et al.*,[9]. In this study, the growth of primary and secondary tumors was significantly increased by CXCR4 expressing cells compared to negative CXCR4 expressing cells. This CXCR4 dependent growth was also shown to be impaired in vitro and abolished in vivo by knocking down the CXCR4 expression in neuroblastoma cells. This further confirmed the survival- promoting properties of CXCR4 in neuroblastoma.

Several methods were used to evaluate the CXCR4 expression in tumors and one of the most commonly used methods is the immunohistochemistry. Immunohistochemical expression of CXCR4 on tumors was demonstrated by many studies looking for its clinical significance as a prognostic marker and potential therapeutic target for the therapy of cancers. There are controversial data regarding the localization of CXCR4 expression on tumor cells. Some studies showed that CXCR4 staining was localized at peripheral or nucleus of tumor cells where as others demonstrated a cytoplasmic staining [15]. Our results showed that CXCR4 expression was predominantly nuclear staining.

Immunohistochemical expression of CXCR4 on primary neuroblastoma tumors was reported before by Russell *et al.,*[16], but none have investigated the expression of CXCR4 in metastatic neuroblastoma tumors. Our results showed that CXCR4 was expressed in both primary and metastatic neuroblastoma samples. High levels CXCR4 were shown to be expressed in advanced stage tumors which were more likely to metastasize to bone marrow. This was consistent with the previous study showing that tumors of advanced stage of neuroblastoma had high CXCR4 expression and increased metastatic capacity to bone marrow[16]. Moreover, high CXCR4 expression connoted poor disease prognosis especially in patients with advanced stage of neuroblastoma.

CONCLUSION

The CXCR4/SDF1 axis is emerging as a great opportunity for tackling cancer metastasis. Here, the CXCR4 was aberrantly and consistently expressed in both primary and metastatic neuroblastoma tumours. This expression connoted unfavourable prognosis and increased metastatic potential to bone marrow. Therefore, the CXCR4/SDF1 axis may represent a potential therapeutic target for the development of new therapy to curb metastasis of neuroblastoma and other CXCR4 expressing cancers.

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