Chemokines in Ewing sarcoma

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Chapter 1

General introduction

EWING SARCOMA

Ewing sarcoma (EWS) is a malignant round cell tumor occurring predominantly in bone in children and young adolescents and to a lesser extent in soft tissue and occasionally organ based [1]. EWS is the third most common primary bone sarcoma with an incidence of three per million in this patient group [2]. It is slightly more common in male than female (1.4:1) and majority of patients are Caucasians [3]. It was first recognized by Lücke in 1866 and afterwards reported by Hildebrand and Marckwald at the end of the nineteenth century [4,5].

Ewing designated new attention to the tumor, popularized it by giving it the entity 'diffuse endothelioma of bone' as a descriptive name and in detail described the morphology as little differentiated round cell tumor which was highly vascularized [6]. The use of intensive chemotherapy, radiotherapy and surgery increased the three year event free survival in patients diagnosed with a localized disease to 75.4% [7]. However, 15% to 30% of the patients have metastases at diagnosis and, together with patients with a recurrent disease, have a poor survival of about 30% [8,9].

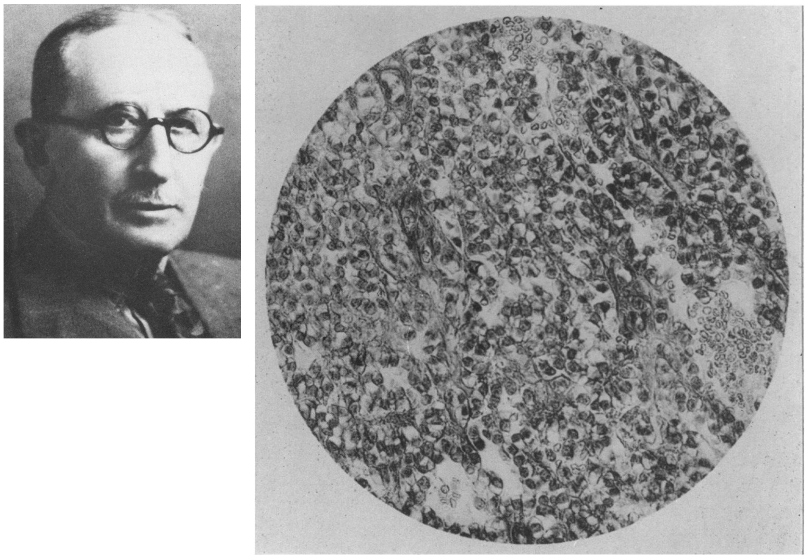

Figure 1: James Ewing and a by him published image of an Ewing sarcoma demonstrating blood sinuses lined by tumor cells, adapted from Ewing J. [6].

CLASSIFICATION OF EWING SARCOMA

Since EWS shows very little differentiation with the exception of some degree of neurogenic differentiation in some cases, the cellular origin of tumor still remains enigmatic. Based on clinical presentation, several clinical entities have been described such as peripheral primitive neuroectodermal tumor (PNET) and Askin tumor. However, in the latest WHO classification all separate clinical entities are unified to EWS characterized by its pathognomonic chromosomal translocation [1]. The translocation fuses at the 5' end the *EWSR1* gene, located on chromosome 22, with a gene of the ETS transcription factor family at the 3' end. The most common 3' end partner is, with 85%, the on chromosome 11 located gene *Friend leukemia*

virus integration site 1 (*FLI1*), followed by the *ETS-related gene* (*ERG*) on chromosome 21 with 10% [10,11]. More sporadic 3' end partners are *ETV1*, *ETV4* and *FEV* [1]. The incidentally detected *FUS-ERG* fusion gene in a EWS patient demonstrates that, like the 3' end partner gene the 5' end partner is interchangable [12]. Gene fusion involving non-ETS gene or genes neither TET nor ETS genes with histopathological features of EWS have been recently reported involving *EWSR1-NFTAC2, BCOR-CCNB3* or *CIC-DUX4* genes. These entities are collectively being grouped as Ewing-like sarcoma [13-15].

TUMOR MICROENVIRONMENT OF EWING SARCOMA

The *EWSR1-ETS* fusion gene is, besides the genetic marker of EWS, also the oncogenic driver [16]. The *EWSR1*, a housekeeping gene, has an RNA binding domain on the 3' end and a transactivation domain at the 5' end with yet not well-defined functions. The 3' end of the ETS gene included the fusion contains a DNA binding domain and acts as a transcription factor. The chimera protein has a preferential binding to GGAA repeats modulating gene expression of nearby genes [17,18]. The fusion protein interferes with various fundamental cellular processes including expression, splicing and protein signaling resulting in a malignant, aggressive growth and highly vascularized phenotype [19-21]. Such a phenotype is often the result of an extensive interplay between the tumor cell and its microenvironment. The tumor microenvironment consists of extracellular matrix, blood vessels, stromal cells, monocytes and leukocytes [22]. The tumor microenvironment is crucial for the tumor to metastasize and for blood vessel growth (angiogenesis) [23]. Main players in facilitating the interaction within the tumor microenvironment are chemokines with at least four major features: 1) attraction of endothelial cells and pericytes leading to angiogenesis; 2) paracrine or autocrine stimulation signaling leading to tumor cell proliferation and 3) increased tumor cell migration and invasion and 4) influence of the immune response.

CHEMOKINES

Chemokines are small chemoattractive proteins, which vary from eight to ten kDa in their molecular weight. Based on the presence of four conserved cysteine residues they can be divided in subfamilies depending on their cysteine pattern as CXC, CC, CX_3C and XC chemokines [24]. The CXC subfamily can be further subclassified as ELR^- and ELR^+ (glutamic acid-leucine-arginine) amino acid motif containing chemokines where ELR^+ (CXCL1, 2, 3, 5, 6 and 8) are angiogenic and ELR- chemokines (CXCL9, CXCL10 and CXCL11) are angiostatic [24,25]. Besides angiostatic, CXCL9 and CXCL10 are T-cell attractants and protein expression levels of CXCL9 and CXCL10 in EWS correlated with increased number of cytotoxic T-cells and a better patient survival [26]. Immunotherapy to stimulate the attraction of immune cells and to improve the immune response in EWS were successfully applied in *in vitro* studies providing a promising ground for novel immunotherapy approaches for EWS patients [27-29].

CXCR4 SIGNALING

Most chemokines and receptors are expressed by specific cells and are involved in some of the four major processes in the tumor microenvironment; angiogenesis, proliferation, metastasis and immune system recognition. Stromal derived factor 1 (SDF-1/CXCL12) and its receptor CXCR4 are exceptions since they are widely distributed across the body and involved

in the regulation of all four main tumor microenvironmental processes. [30,31]. Moreover, overexpression of CXCR4 receptors in tumors is frequently associated with increased metastatic propensity and poor clinical outcome [31-33]. Various downstream signaling pathways of CXCR4 mediate its diverse functions (**Figure 1**). CXCL12 binding to CXCR4 leads to activation of the receptor which then activates an associated G protein leading to the activation of the migration associated protein kinase C and cofilin pathway and the proliferation stimulating PI3K-Akt and RAS-MAPK-ERK pathways [30,34]. After ligand binding, CXCR4 is phosphorylated at the C-terminus and recruits Beta-arrestins. Depending on the type of recruited beta-arrestin, these either desensitize the G protein activation or transduce the activation signal towards RAF-MEK-ERK pathway [35]. The desensitization of CXCR4 activation is followed by internalization of the receptor leading to either recirculation or degradation [36,37]. Endocytosis and recycling of the receptor are vital for proper functioning of CXCR4 [38]. Recent reports suggest that the internalization of the receptor and its downstream signaling are connected to each other [39,40].

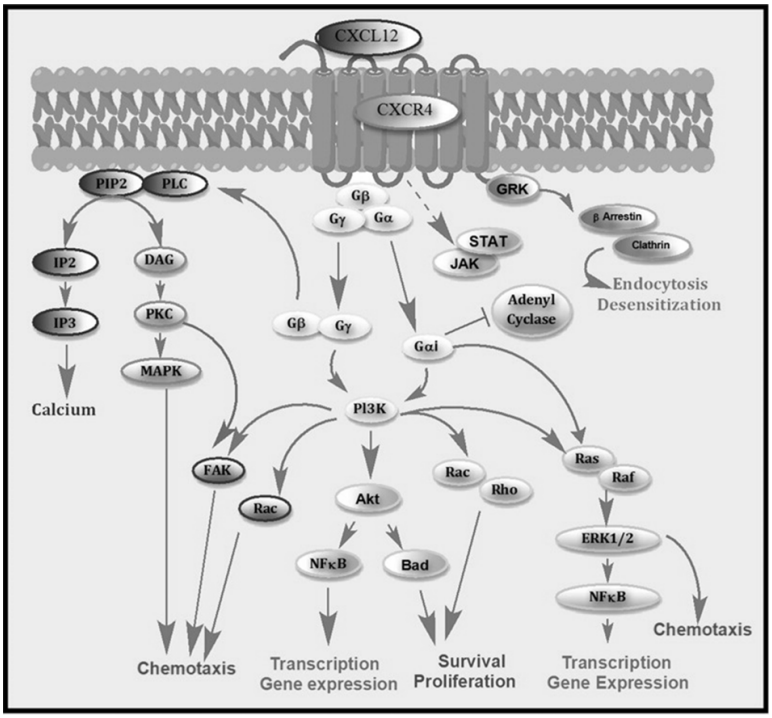

Figure 2: CXCR4 downstream signaling, adapted from Lauren E. Woodard and Sridhar Nimmagadda *et al.*[41].

WHOLE TRANSCRIPTOME ANALYSIS OF CHEMOKINES

The chemokine field is still in development and novel chemokines and chemokine receptors have recently been identified [42-44]. In addition, alternative splicing of chemokines and chemokine receptors forms novel isoforms with other or additional functions [45-48]. Whole transcriptome sequencing, next generation sequencing of the RNA, of tumors elicits the identification of novel splice variants. Whole transcriptome sequencing is an unbiased approach

since it uses adapters to sequence the complete RNA, both coding and non-coding. When analyzing the mapped paired-end novel splice variants and their sequence can be determined. In addition it can be used to quantify the splice variants expressed.

AIM OF THE STUDY AND OUTLINE OF THIS THESIS

The aim of this study was in depth investigation on the presence of chemokines and their role in the tumor microenvironment of EWS and the clinical usability of the chemokines as predictive and/or prognostic markers in EWS.

Detailed characterization of EWS and the effect of the fusion protein on the tumor cellular processes at DNA, RNA and protein level is needed to unravel the interactions with its tumor microenvironment. When this interplay is better defined it might lead to identification of new therapeutic targets and predictive and prognostic candidate markers. Recent advances in the sequencing has helped to fulfill this characterization of EWS at genome, epigenome and transcriptome level. In **Chapter 2** a comprehensive compilation/overview is given of all the reported sequencing studies in EWS. The results of these studies were put in clinical perspective by linking them with treatment efficacy of existing therapies against EWS. In additional, novel potential targeted therapies and immunotherapies were reported which were identified or strengthened by the sequencing results.

The role of chemokines in the immune microenvironment in EWS was partially studied previously by us. We extended the understanding of EWS its immune microenvironment with the role of the T-cell attracting chemokine CCL21 in EWS (**Chapter 3**). CCL21 is, besides its T-cell attracting ability, of interest since CCL21 activation-based immunotherapy is tested in a clinical trial for non-small cell lung cancer.

The highest expressed chemokine receptor in EWS found so far is CXCR4. In **Chapter 4** we focused on the role of the CXCR4 network in EWS by investigating the *in vitro* and *in vivo* expression of the *CXCR4-CXCR7 axis* genes and of the two described isoforms *CXCR4-1* and *CXCR4-2*. The obtained levels were correlated to clinical parameters in two independent cohorts.

Since the EWSR1-ETS fusion protein alters splicing, alternative splicing of CXCR4 was analyzed by whole transcriptome sequencing. This has led to identification of the 2 new CXCR4 isoforms (**Chapter 5**). Detailed characterization of the function of the two novel isoforms were performed and presented.

CXCR4 has various signaling pathways and CXCR4 localization and protein dynamics is important in regulating its activity. Earlier EWS studies demonstrated contradictory results between RNA and protein expression in metastases. Since only cell membrane located CXCR4 is able to be bound and activated by a ligand, we studied the CXCR4 cell membrane expression in EWS by using a peptide-based method (**Chapter 6**). With this method CXCR4 cell membrane expression in EWS cell lines could be determined qualitatively and quantitatively. As the receptor was internalized upon binding the peptide, we synthesized and validated *in vitro* and *in vivo* a CXCR4 endocytosis tracker based on the previous used peptide combined with a FRET (Förster resonance energy transfer) dimer, to track the internalized receptor and study the endocytosis of CXCR4 (**Chapter 7**). In the FRET dimer Cy5 and Cy3 fluorophores were connected by a disulfide bond which can be reduced intracellularly. Reduction disrupts the FRET ability of the dimer and the resulting difference in fluorescence intensity can be measured.

In **Chapter 8** the results are summarized and the future perspectives are discussed.

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