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Drugs targeting the bone microenvironment: new therapeutic tools in Ewing's sarcoma?

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Abstract

Introduction. Ewing's sarcoma (ES) is the second most frequent malignant primary bone tumour in children, adolescents and young adults. The overall survival is 60-70% at 5 years, but still very poor for patients with metastases, disease relapse, or not responding to chemotherapy. For these high risk patients, new therapeutic approaches are needed beyond conventional therapies (chemotherapy, surgery and radiation) such as targeted therapies.

Areas covered. Transcriptomic and genomic analyses in ES have revealed alterations in genes controlling signalling pathways involved in many other cancer types. To set up more specific approaches, it is reasonable to think that the particular microenvironment of these bone tumours is essential for their initiation and progression, including in ES. To support this hypothesis, preclinical studies using drugs targeting bone cells (bisphosphonate zoledronate, anti-RANKL strategies) showed promising results in animal models. In this review, we will discuss the new targeted therapeutic options in Ewing's sarcoma, focusing more particularly on the ones modulating the bone microenvironment.

Expert opinion. Targeting the microenvironment represents a new option for patients with Ewing's sarcoma. The proof-of-concept has been demonstrated in preclinical studies using relevant animal models, especially for zoledronate which induced a strong inhibition of tumour progression in an orthotopic bone model.

1. Background

Ewing's sarcoma (ES) first described by James Ewing is a high-grade neoplasm and represents the second most common primary bone malignancy in both children and adults (1). With a peak incidence at 15 years, this disease accounts for 2% of childhood cancers (2). Ewing's sarcoma is defined as a bone tumour which may occur at any site within the skeleton but preferentially affects the trunk and the diaphysis of long bones (3). Less commonly, it arises in extraskeletal soft tissues (15%). It is characterized by a rapid tumour growth and extensive bone destruction that can result in bone pain and pathological fracture (4). At histological level, ES appears as small, poorly differentiated, round tumour cells (Figure 1) positive for CD99 staining (5).

Ewing's tumours are characterized by the occurrence of a typical chromosomal translocation arising in cells from mesenchymal origin (6) that fuses the EWS gene on chromosome 22q12 to a member of the Erythroblast Transformation Sequence (ETS) transcription gene family, most commonly Fli-1, on 11q24 in 85% of cases (7,8). This translocation leads to the production of the oncogenic fusion gene EWS-Fli1, an aberrant transcription factor that promotes tumourigenicity (9-11). Recently, a new fusion was observed between BCOR (encoding the BCL6 co-receptor) and CCNB3 (encoding the testis specific cyclin B3) on the X chromosome in individuals diagnosed with small round cell tumours of bone, possibly Ewing's sarcoma, but which lack the canonical EWSR1-ETS translocation (12). Numerous biological pathways such as those involving Insulin-like Growth Factor Receptor (IGFR), Platelet Derived Growth Factor Receptor (PDGFR), Vascular Endothelial Growth Factor Receptor (VEGFR), Sonic HedgeHog (SHH) pathway activation, Wnt, Transforming Growth Factor (TGF)β Receptor II pathway inhibition, are modulated by EWS-FLI1 activity leading to proliferation, angiogenesis, immune system escape, metastatic potential, and treatment resistance that contribute to the ES malignant phenotype (13).

2. Medical need

The existing treatments for Ewing's sarcoma patients are not effective enough especially for patients with disease relapse because they are resistant to conventional

chemotherapy, or for patients with metastatic disease. Moreover, the current survival rate (around 70% at 5 years for localized disease, and less than 30% for high risk patients) have not evolved since three decades. In addition, even for the good responders, the current treatments induce much adverse side effects. For all these reasons, there is an urgent need to define new therapeutic targets for Ewing's sarcoma patients and besides the tumor cells themselves, targeting the bone tumor microenvironment appears to be promising.

3. Existing treatments

Before the 1980's, amputation was the only therapeutic option with a 5 years survival of less than 20%. Introduction of chemotherapy in the 70ies has incredibly modified their prognostic with the 5 years event free survival (EFS) rate of localized tumours being around 65% and overall survival rate close to 75%. However, the survival rates decrease to 15-25% when metastases are detected at diagnosis, or for patients presenting resistance to treatment or relapsed disease. Moreover, a survival plateau seems to have been reached for the past three decades with conventional therapies (14).

The improvement of poly-chemotherapy allowed more limited surgery with limb salvage, but in about 20% of cases, bone sarcomas have already disseminated at the time of diagnosis. The site of distant metastases is most often lungs, then the skeleton. Although ES patients with lung metastases have an overall survival of 45% at 5 years, those with bone or bone marrow metastases have very poor prognosis with less than 25% OS at 5 years. In the past, when therapy was limited to local control (surgery), nearly all those patients who initially appeared to have a localized tumour developed distant metastases. Ewing's sarcoma thus has to be considered as a systemic disease, requiring systemic treatment, i.e. combination chemotherapy, as a rule. However, systemic therapy can never replace definitive local control by surgery and/or radiotherapy. The therapy of Ewing's sarcoma therefore requires a combination of surgery, high-intensity chemotherapy and radiotherapy. The currently active protocol for Ewing's tumours is the EUROpean Ewing tumour Working Initiative of National Groups 99 protocol (EURO-E.W.I.N.G. 99). It consists in combination of chemotherapy with or without peripheral stem cell transplantation, radiation therapy, and/or surgery (Figure. 2).

3.1. Surgery

In Ewing's tumours, radical local treatment is also associated with a favourable prognosis. Appropriate surgical excision with tumor free margins has been shown to be an important prognostic factor (table 1) (15). However, response to chemotherapy is also of considerable prognostic relevance, and tumours which cannot be completely removed may receive additional radiotherapy for better satisfactory local control (15). Definitive surgery ought to be given following neo-adjuvant chemotherapy for two main reasons: (i) tumour resection will be better when following pre-operative chemotherapy which limits tumour development and allows the surgeons to remove the primary tumour without mutilating surgery; (ii) the relevant histological response can thus be assessed to set up the optimal post-operative treatment strategy (table 2). To date, this histological response to neo-adjuvant chemotherapy has proven to be the most important prognostic factor for Ewing's sarcoma patients. Owing to neoadjuvant chemotherapy and advanced surgical procedures, more than 80% of patients can have limb-sparing surgery.

3.2. Radiotherapy

Whenever surgery is marginal or intralesional and whenever histological response is found to be poor (> 10% of viable tumour cells), post-operative radiotherapy is indicated as Ewing's tumours respond well to radiotherapy (16). Dose recommendations for post-operative radiotherapy are based on histological response and surgical margins. They range from 45 Gy in case of good response and tumour-free surgical margins, to 55 Gy for poor response and contaminated margins.

3.3. Chemotherapy

Patients with Ewing's sarcoma have to be treated in experienced centres within controlled clinical trials. Ewing's tumours are sensitive to cytotoxic agents, especially alkylating substances such as ifosfamide and cyclophosphamide, anthracyclins (doxorubicin) and other agents such as vincristin, actinomycin D, and the topoisomerase II inhibitor etoposide. Combining cytotoxic substances with complementary mechanisms of action has made a significant contribution to improve the disease free survival rate. The EURO-E.W.I.N.G. 99 protocol used in many European countries provides for a 6-cycle of VIDE

(vincristin, ifosfamide, doxorubicin and etoposide) as induction chemotherapy for all patients (Figure 2). The response of patients to initial chemotherapy is determined at surgery on the resection piece (table 1). This is followed by surgery and/or radiotherapy for local control. Following local therapy, the patients receive risk-adapted consolidation treatment. The risk groups are determined based on several criteria: histological response, tumour size, dissemination status at diagnosis. Characteristics of standard risk are a localized tumour, good clinical and histological response to pre-operative chemotherapy and/or initial volume < 200 ml. These patients will be randomized to receive either an ifosfamide containing three-agent combination (VAI: vincristin-actinomycin D-Ifosfamide) or a cyclophosphamide (C) containing three-agent combination (VAC). On the contrary, characteristics qualifying for high risk are poor clinical/histological response to induction chemotherapy, large initial tumour volume (> 200 ml) when not operated, and/or pulmonary metastasis at diagnosis. Such patients will be randomized to receive either consolidation of standard chemotherapy (VAI), combined with radiation to lung metastases as needed, or busulphan/melphalan-containing high dose therapy and autologous stem cell re-infusion. Patients with bone metastases or multiple metastases at diagnosis receive high dose therapy and/or experimental treatment following induction (Figure 2).

3.4. Relapse treatment

The majority occurs with the 1.5 years following diagnosis. In Ewing's sarcoma, the prognosis in case of an early relapse within 2 years from diagnosis and in multi focal relapse is poor (< 10%). Prognosis is more favourable in focal late relapse. Successful relapse therapy requires good tumour response to relapse chemotherapy and radical local treatment (2).

The main problems of these treatments are side effects and late sequelae. For surgery and radiotherapy (local therapies), acute side effects include pain and a high risk of secondary wound infection, especially in patients who are unusually immune-compromised from the preceding chemotherapy. Surgery may be associated with considerable late sequelae depending on the extent of surgery. Radiotherapy regularly induces skin and mucosal irritation and possibly gastrointestinal problems. Late sequelae may also concern growth or development impairment, development of fibroses, impaired lymph flow and pigmentation changes in the affected region.

Chemotherapy could induce acute haematological toxicity such as leukocytopenia followed by immunosuppression and an increased risk of infection, thrombocytopenia and anaemia. Other side effects such as nausea, vomiting, and mucositis could also be observed. Alkylating agents such as cyclophosphamide and ifosfamide can also induce nephrotoxicity and fertility impairment, especially in men. Another side-effect is the development of peripheral neuropathy due to high cumulative doses of vinca-alkaloids. In addition, actinomycin D may induce hepatotoxicity.

However, secondary malignancies occurring ten years or later after completion of therapy represent the most serious side effect of chemo- and radio-therapy, especially with the use of chemotherapeutic agents acting on transcription (17).

4. Market review for chemotherapy and targeted therapy in cancer

In France, the number of patients treated by chemotherapy is increasing: more than 260 000 patients received a chemotherapy protocol in 2010 (+ 20 % as compared to 2005). The number of patients treated with chemotherapy grows faster than the number of new cancer cases: indications of chemotherapy concern an increasing proportion of cancers. From January 2004 to April 2012, 38 new anticancer drugs that obtained a first European MA in oncology are available in France. Targeted therapies represent 42% of these molecules. In 2010, cancer accounted for 54% of total costs reimbursed with an expenditure amounting to 1,060,145,178 euro. In 2010, 61% of the costs are focused on targeted therapies, showing that over the years, the proportion of conventional cytotoxic molecules has decreased in favour of targeted therapies. This change of cancer use is due to increased knowledge on the biology of cancer cells and development through active research for new molecules.

These new therapeutic approaches are actively explored given the main side-effects observed with current therapies (chemo- and radio-therapy). In addition, these new options should especially benefit high-risk patients, to increase long-term survival by decreasing metastases development and preventing drug resistance.

5. Current research goals

Several strategies are currently under development (18), targeting the fusion protein EWS-FLI1 specific of ES or its downstream activated pathways, or more common target therapies used for other cancers, non specific for Ewing's sarcoma.

5.1. EWS-FLI1 inhibition in Ewing's sarcoma

The fusion protein EWS-Fli1, exclusively expressed in ES tumour cells, represents an ideal target to specifically treat ES without affecting normal cells. Inhibition of cell proliferation and tumour growth in ES xenografts can be achieved by decreased EWS-FLI1 expression induced by antisense oligonucleotides (19) or RNA (20), or small interference RNA (siRNA) administered with nanoparticules (21). However, pharmacological delivery of these large molecules remains problematic in patients. An alternative strategy is to target the interaction between EWS-FLI1 and its partner proteins in the transcriptional complexes in order to inhibit EWS-FLI1 function. For example, YK-4-279 inhibits EWS-FLI1/RNA helicase A (RHA) interaction and induces apoptosis and tumour regression in ES models (22). Recently, mithramycin was found to be the top candidate to inhibit EWS-FLI1 activity after high throughput analysis of 50000 compounds, and to demonstrate ESFT antitumor activity both in vitro and in vivo (23).

Trabectedin is an alkylating agent which inhibits EWS-FLI1 demonstrating an increased efficacy in ES as compared to other paediatric sarcomas (e.g. osteosarcoma (OS), rhabdomyosarcoma) (24). In children and adolescents, trabectedin used in compassionate phases I/II trials shows one 6 months complete response (CR) and stable diseases (SD) in ES (25) with an acceptable tolerance in paediatric phases I/II (thrombopenia, reversible hepatic toxicity) (26). This anti-tumour effect can be increased by combining EWS-FLI1 inhibition with antisens oligonucleotide and EWS-FLI1-modulated pathways (e.g. the mTOR pathway) as revealed by apoptosis analysis and by *in vivo* tumour regression (27).

5.2. Ongoing targeted therapies for Ewing's Sarcoma

5.2.1. Inhibition of growth factor signalling pathways

Most of the signalling pathways are involved in the regulation of cell proliferation and apoptosis resistance. They are mediated by proteins with kinase activity [tyrosine kinase (TK) or serine kinase (SK)], present at the tumour cell surface, in the cytoplasm or in the nucleus. These proteins may be inhibited by monoclonal antibodies directed against extra-membrane receptor or by small molecules which inhibit the intra-cellular kinase domain.

- *IGF-1R/PI3K/AKT/mTOR pathway*: the Insulin-like Growth Factor (IGF)-1R pathway plays an important role in paediatric cancers, including ES (28). This tumour has a peak incidence at puberty, suggesting a role of growth hormone and IGF-1. As others, IGF-1R pathway activates the downstream PI3K/Akt/mTOR pathways, stimulates ES cell survival and angiogenesis through Hypoxia Inducing Factor (HIF)-1α and Vascular Endothelial Growth Factor (VEGF) secretion. With different anti-IGF-1R monoclonal antibodies, children and adolescents suffering of relapsed or refractory ES had stable disease in phase I trials (29) and 10-15% of objective responses in paediatric/adult phase II trials (30). Predictive factors of the therapeutic response to IGF-1R antibodies remain mostly unknown. The reduced activity of the IGF system might associate with tumour progression and poor response to treatment (31). As a consequence, high expression levels of IGF-1R, insulin receptor (IR) and IGF-1 mRNAs is associated with increased survival, and high circulating IGF-1 levels with low progression risk (32). Unfortunately, the median duration of ES response was only 5-7 months (30), probably because tumour cells escape IGF-1R inhibition, through AKT or other signalling pathway activation such as mTOR or TK receptors. These observations lead to consider either combination of monotargeted inhibitors or multitargeted ones.

- mTOR is an intra-cytoplasmic serine/threonine kinase regulated by AKT. In paediatric ES, mTOR represents a promising target as the overexpression of the phosphorylated form of mTOR is correlated with survival (32). The mTOR inhibitor rapamycin was first used in children to prevent graft rejection. Paediatric phase I trials using the two mTOR inhibitors everolimus and temsirolimus have shown a good tolerance profile (33,34). A double blind phase III study comparing ridaforolimus against placebo (SUCCEED trial) in sarcoma treatment maintenance after stabilisation or regression

under chemotherapy, have included 50 bone sarcomas and showed an increased progression free survival (PFS) with mTOR inhibitor (35). Concerning combination of monotargeted inhibitors, strategies targeting simultaneously the IGF-1R/PI3K/AKT/mTOR pathway at several levels are currently evaluated. For example, a phase I-II using Ridaforolimus combined with the anti-IGF1R antibody dalotuzumab is ongoing in children in Europe and US (NCT01431547). Dual PI3K/mTOR inhibitors are also studied in adult phase I trial and dual mTOR/DNA-PK inhibitor (CC-115) in adolescent/adult phase I trial (NCT01353625).

- *Multi-target inhibitors:* imatinib mesylate inhibiting PDGFR, c-KIT, and BCR-ABL is a good candidate for Ewing's sarcoma therapy. Indeed, high expression of c-KIT and PDGFR is observed in ES and associated with low EFS but not with poor response to chemotherapy (36). Imatinib appeared to exert an anti-ES activity *in vitro* and in xenograft models (37). However, expression of imatinib targets is not sufficient to confer drug sensitivity (38). Several phase II trials have shown stabilisation of bone sarcomas and among them 3 out of 20 Ewing's sarcoma with a median Progression Free Survival inferior to 2 months (39). In a paediatric phase II trial from the Children's Oncology Group (COG), only one ES patient out of 24 had partial response (40). Preclinical data showed increased anti-tumour activity of imatinib when combined with doxorubicin and vincristine in ES (41). Dasatinib inhibits Src and BCR-ABL and shows *in vitro* cytostatic and anti-migration effect but no apoptosis in ES (42). Dasatinib was tested in a paediatric phase I trial showing that its pharmacokinetic is similar in children and adults (43). The efficacy of sunitinib, an inhibitor of Flt3, c-KIT, PDGFR and VEGF was observed in *in vivo* models of most paediatric tumours, including 4 out of 5 ES xenografts (44). In a paediatric phase I trial, haematological and cardiac toxicities were observed with sunitinib in children previously treated with anthracyclins (45). Pazopanib, an inhibitor of VEGFR1-3, PDGFRα/β, and c-KIT used in monotherapy is active in paediatric *in vivo* ES models, considering EFS (46). A phase II study of pazopanib in bone sarcoma is ready to begin in Europe.

5.2.2. Cell growth inhibition

Aurora A plays a crucial role during mitosis. The Aurora A inhibitor MLN8237 is associated with prolonged complete response in *in vivo* Ewing's sarcoma models (47). In paediatric phase I trials, two Aurora A inhibitors, are in development: MLN8237 (NCT01154816) and AT9283 (NCT00985868 and NCT01431664).

MDM2, an oncoprotein that negatively regulates p53, is overexpressed in p53 wild-type cancers. The MDM2 inhibitor nutlin-3 activates p53 signalisation pathway leading to important tumour regressions in p53 wild-type ES through apoptosis. This effect can be increased by either NF-κB inhibition (48) using TNF alpha (49), or HDAC inhibitors (50). An adult Phase I study using the oral MDM2 inhibitor RO5503781 is ongoing in solid cancers (NCT01462175).

5.2.3. Resistance to cell death

Resistance to apoptosis is a key element in tumour progression and chemoresistance. The mechanisms involve increased survival signals (growth factors/TK receptors, downstream pathways), anti-apoptotic molecule overexpression (Bcl-2, Bcl-XL, FAK in osteosarcoma), pro-apoptotic molecule under-expression, or resistance to cell death receptors involving the systems Fas/FasL (Fas ligand) or TRAIL (TNF-Related Apoptosis Inducing Ligand). The BCL2 inhibitor navitoclax is developed in adult refractory tumours in combination with docetaxel, with acceptable toxicity and few responses (2 Partial Responses, 5 Stable Disease) (51). TRAIL induces tumour cell apoptosis in ES mice models, decreases osteolysis and prolongs survival (52). Combination with Imatinib further increased TRAIL effect on tumour growth and metastases in *in vivo* ES models (53). IAPs (inhibitor of apoptosis proteins) inhibit caspase-dependent apoptosis. X-linked IAP antisens oligonucleotide (XIAP ASO-AEG35156) decreases XIAP in paediatric tumour cell lines including ES (54).

Resistance to cell death may also be induced by replicative immortality through restoration of telomerase activity in cancer cells. Telomerase activity is present in 100% of ES metastases, but only in 12% of primary ES tumours and associates with shortened telomeres and decreased patients survival (55). It is interesting to note that telomerase activity is induced by EWS-FLI (56). The telomerase inhibitor TMPyP4 decreases telomerase

enzyme activity, but its effect on cell growth inhibition depends on the cellular context (57). Telomerase activity is inhibited by imatinib, doxorubin or radiation in ES (58-60).

5.2.4. Inhibition of the metastatic phenotype

Each step of the metastatic process might be targeted by different therapeutic classes. For example, invasion of the host extracellular matrix is mediated by the Notch/Hes1 pathway (61). In ES, Notch is involved in neural differentiation, proliferation and apoptosis, but its inhibition in established tumour models had poor anti-tumour effect (62).

In addition, the loss of intercellular and cell/extracellular matrix contacts may lead to anoikis, an apoptotic cell death linked to the Src/PI3K/AKT and Wnt/β-catenin/NF-κB pathways. Tumour cell survival in bloodstream is associated to resistance to anoikis and ability to escape the immune system. A phase I of LY2090314 (GSK3 inhibitor)/pemetrexed/carboplatin combination is ongoing in adults with progressive solid tumours, with good tolerance and restoration of β-catenin expression (63).

In the case of primary bone tumours, lungs represent the preferential site for metastases dissemination. Chemokines and adhesion molecules are key factors for circulating tumour cells to form metastases, the extravasation process towards the target tissue depending on MMP-2 and MMP-9 production and activity. CXCR4 is one of the most important chemokine involved in dissemination mechanisms. CXCR4 inhibitors are used in patients to treat HIV infection and to mobilise hematopoietic stem cells (AMD3100). Adhesion and survival to the novel microenvironment depends on Erzin / β4-integrin / PI3K pathway and Fas/FasL mediated resistance to apoptosis (64).

Isolated cells or micrometastases can enter in a prolonged survival state called “dormancy” that might be responsible for late metastatic recurrences or resistance to cytotoxicity. Dormancy depends on αVβ1 integrin activation of NF-κB, on the anti-apoptotic molecule Bcl-XL and on the ratio ERK/p38-MAPK (65). Cilengitide is the unique integrin inhibitor currently developed in children with high affinity for αvβ3/αvβ5. It disorganises the cytoskeleton and the tight junctions inducing the detachment of endothelial and tumour cells, and also induces apoptosis and inhibits angiogenesis (66). A paediatric phase I trial in brain tumours showed similar pharmacokinetic as compared to adult and no dose limiting

toxicity (66). A paediatric phase I trial in combination with irradiation is ongoing for children and adolescents with diffuse brainstem high grade gliomas (CILENT-0902, Trial NCT01165333).

5.3. Modulation of the anti-tumour immune response

Interferon (IFN) present in the pro-inflammatory microenvironment of ES is more often detected in metastasis than in primary tumours. It participates to neoangiogenesis by the secretion of VEGFR and to the metastatic potential by MMP-9 secretion (67). The preclinical association of IFN with ifosfamide decreases both VEGFR and MMP-9 and inhibits tumour growth, but at doses that cannot be reached in humans (68). The increase in pro-inflammatory type I cytokines or chemokines in the tumour correlates with cytotoxic CD8(+) T-lymphocyte infiltration in the tumour which is associated with tumour progression (69). *In vivo*, an elevated rate of C-reactive protein, white blood cell count and an important vascularisation are associated with tumour macrophages infiltration and decreased survival in ES patients (70). In ES patients, fever can also be considered as a prognostic factor whatever the metastatic status. Celecoxib, a COX2 inhibitor prevents pulmonary metastases in a murine model of ES without effect on the primary tumour and its vascularisation (71).

Another target concerns the gangliosid GD2 which is expressed at the cell surface of ES (72). This neuroectodermic marker can be targeted by an anti-GD2 monoclonal antibody which, when combined with Interleukin-2 and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) has significantly increased metastatic neuroblastoma survival (73).

Beyond these non specific targeted therapies, new drugs targeting the bone tumor microenvironment require special attention, and could be proposed as an adjuvant to conventional therapy: chemotherapy and surgery.

6. Scientific rationale for targeting Bone microenvironment

Interest has recently dramatically increased on the importance of the microenvironment, also called “the bone niche” in the progression of bone sarcoma and establishment of resistance processes to conventional therapies. Therefore, new therapeutic options

targeting hypoxia, angiogenesis or bone microenvironment have been extensively studied at the preclinical level, the more promising being now proposed in clinical trials. This review will describe the most recent developments in such therapeutic options for Ewing's sarcoma patients.

Ewing's sarcoma is characterized by extensive bone destruction, mainly due to osteolysis (Figure 3). ES cells are unable to degrade the bone matrix and accordingly, osteoclast activation and subsequent bone resorption might be responsible for the clinical features of bone destruction (4). Bone degradation is under the control of osteoclasts whose differentiation and activation is mainly mediated by Receptor activator of NF-kB Ligand (RANKL), a member of the Tumour Necrosis Factor (TNF) super-family (TNFSF11) after its binding to its receptor RANK expressed at the surface of mature osteoclasts and osteoclast precursors (74). Osteoprotegerin (OPG) acts as a decoy receptor inhibiting osteoclast formation, function and survival by preventing the binding of RANKL to its receptor RANK (75).

Interaction between tumour cells, tumour-derived humoral factors and the bone marrow in the bone niche has been shown to be essential for bone tumour initiation and promotion. **Therefore, targeting the bone microenvironment and particularly osteoclast activation may represent a promising adjuvant strategy for the treatment of bone tumours including ES.** Indeed, a vicious cycle between osteoclasts, bone stromal cells/osteoblasts and cancer cells has been hypothesized during the progression of primary bone tumours (76, Figure 4). Tumour cells produce osteoclast activating factors such as Interleukin-6, TNF-α or ParaThyroid Hormone-related Peptide (PTH-rP) which induce osteoclast differentiation and activation. When they resorb bone, osteoclasts enable the release of growth factors stored in the bone matrix (TGF-β, IGF-1, PDGF…) that in turn activate tumour cell proliferation (76). Accordingly, inhibition of osteoclast activity represents a promising approach to block the vicious cycle, thereby indirectly limiting local cancer growth.

Two main approaches can be proposed to target bone resorption is ES: (i) inhibition of osteoclast functions by using bisphosphonates, or (ii) direct inhibition of RANKL. BP's such as pamidronate (77) and minodronate (78) had been shown to have a direct effect on ES cells therefore combining an indirect effect through inhibition of bone resorption and a direct cytotoxic effect. Zoledronate (or Zoledronic acid, Zometa from Novartis Pharma), a nitrogen-containing BP of the third generation with hetero-cycle is one of the most potent

inhibitor of bone resorption (79). Based on promising effects observed in preclinical studies in osteosarcoma, zoledronate has been proposed for therapeutic use in ES. Moreover, zoledronate combination to first line OS chemotherapy (methotrexate or adriamycin / platinium / ifosfamide-based chemotherapy) is currently tested in the French randomised phase III trial (OS2006, NCT00470223). In *in vivo* ES models, zoledronate alone induces inhibition of tumour progression in the bone environment, an effect on tumour induced in soft tissue being only obtained when combined with ifosfamide (80, 81). However, it must be noted that in juvenile murine models, zoledronate decreases enchondral bone growth in a reversible manner (82).

Among the factors involved in the regulation of bone remodelling, attention mainly focuses on the molecular triad OPG/RANK/RANKL (75). Concerning the inhibition of RANKL, very little is known in ES. If we compare with osteosarcoma, RANKL tumour expression in OS patients associates with poor response to pre-operative chemotherapy, high expression with decreased survival, and high TRACP5b plasma levels (osteoclast activity marker) with metastases occurrence (83,84). The naturally occurring RANKL decoy receptor, OPG offers considerable promises as a new modality for treating osteolysis associated to bone tumours (85-87). Indeed, increased expression of RANKL has been observed in osteolytic malignancies such as breast cancer and multiple myeloma (88, 89). Concerning primary bone tumours, the inhibition of RANKL activity by OPG induced a significant therapeutic effect on bone lesion and tumour development in two preclinical models of osteosarcoma in mice (POS-1) and in rats (OSRGa) (90). This effect was also confirmed by using the soluble form of the RANKL receptor, RANK-Fc (91) or by the RNA interference strategy targeting RANKL (92). Less data are available in ES, but the presence of RANKL has been reported in patient biopsies by two independent studies (93, 94) suggesting that it may represent interesting therapeutic target (figure 5). However, RANKL inhibition has no effect in ES cells *in vitro* suggesting that the decrease in primary bone tumour progression seen in preclinical models is due to indirect effect through inhibition of bone resorption, limiting the release of pro-tumoural growth factors stored in the bone matrix. In addition, modulation of RANKL by VEGF165 may be one of the mechanisms responsible for the osteolytic process induced by Ewing's sarcoma cells (95). VEGF165 may, therefore play an important role in modulating RANKL gene expression in the bone marrow microenvironment during the metastatic process, thereby contributing to tumor induced bone lysis. At the clinical level, the use of

denosumab, a humanised monoclonal antibody (IgG2) with high affinity and specificity against RANKL (96) is promising for primary bone tumour patients including ES, as it shows interesting results in several cancers with bone metastases (97). A phase II safety study of denosumab in subjects ≥12 years with recurrent and unresectable bone giant cell tumour is ongoing (NCT00680992) (98).

DDK1 inhibitors interfere with the Wnt pathway and bone metabolism. Monoclonal antibody BHQ880 anti-DKK1 might restore bone formation but without direct anti-tumour effect. BHQ880 is currently in adult phase I/II trials for multiple myeloma, alone (NCT01302886; NCT01337752) or associated with zoledronate (NCT00741377). Preliminary data on ES xenograft model show significant decreased tumour progression likely due to indirect effect on the bone microenvironment cells (personal observations).

The Ewing's sarcoma micro-environment not only includes bone cells, but also angiogenesis and hypoxia.

6.1. Angiogenesis and vasculogenesis inhibition

Angiogenesis is defined as new capillaries formation from preexisting vessels and vasculogenesis as new vessels from bone marrow-derived progenitor cells (99). Platelet Derived Growth Factor Receptor (PDGFR), VEGF, VEGF Receptor (VEGFR) and their downstream pathways (PI3K/AKT) are implicated in angiogenesis, VEGFR and Notch (DLL4) in vasculogenesis. PDGFR and VEGFR are overexpressed in ES and associated with poor prognosis (100, 101). After cytotoxic chemotherapy, bone marrow progenitor cell number increases, favouring expansion of residual tumour cell or micrometastases. Hypoxia increases these phenomena, especially through induction of HIF (Hypoxia Inducing Factor)-1α expression, a factor associated with increased resistance to treatment in ES (102). HIF1α expression is also induced by PI3K/AKT/mTOR, RAS/MAPK pathways and calcium signalling. HIF1α plays additional specific role in ES as it modulates EWS-FLI expression in Ewing's sarcoma (103) and modulates bone sarcoma cell proliferation and apoptosis (104)

Bevacizumab is an anti-VEGF IgG1 monoclonal antibody which inhibits VEGF/VEGFR-1 and VEGFR-2 interactions and VEGF-dependant angiogenesis. A good tolerance has been observed in children/adolescent with few side effects (proteinuria, thrombotic risk). A phase II trial (COG-AEWS0521, NCT00516295) of bevacizumab combined with vincristin/topotecan/cyclophosphamide in first recurrent ES showed good tolerance.

Cediranib is another VEGFR inhibitor which delays tumour growth in all *in vivo* ES models studied (105), its effect being further increased when combined with mTOR inhibitor (rapamycin) but not with chemotherapy (vincristin, cyclophosphamide, cisplatin) (105).

6.2. Hypoxia

Concerning hypoxia, mTOR and topoisomerase I inhibitors decrease HIF-1α accumulation leading to an important anti-tumour effect when combined (106). A combination rapamycin with irinotecan is ongoing in a paediatric phase I trial in France [RAPIRI, NCT01282697, sponsored by the SFCE (Société Française des Cancers de l'Enfant)].

6.3. Competitive environment

In view of the previous data on bone tumor environment, two main drugs could be proposed to target bone microenvironment in Ewing's sarcoma: zoledronate, a bisphosphonate of the third generation and (ii) denosumab, a fully humanized anti-RANKL antibody (Amgen Inc). These drugs are already tested in clinical trials but in other indications: bone metastases (both), multiple myeloma (both), giant cell tumours (denosumab), osteosarcoma (zoledronate). Even if they target different levels of bone resorption in the bone microenvironment (at cellular level for zoledronate and molecular level for denosumab), they both induce strong inhibition of bone degradation, a relevant target for bone tumours. They could therefore be used as adjuvant treatment in combination with chemotherapy, to prevent tumour development in primary bone site but also bone metastases formation, the worst prognosis factor for Ewing's sarcoma patients.

6.4. Potential development issues

Both zoledronate and denosumab are therefore potentially interesting tools for therapeutic application in Ewing's sarcoma. However, they both induce adverse side-effects that must be considered. Because zoledronate is a potent inhibitor of bone resorption, it may induce adverse side effects on bone and craniofacial growth during childhood and adolescence, the time of Ewing's sarcoma development. Recent preclinical studies in young mice have reported an irreversible delay of long bone growth (82), and reversible delay in tooth eruption (submitted). Intravenous bisphosphonates used in oncology including

zoledronate have been associated with acute phase response, hypocalcaemia and secondary hyperparathyroidism, musculoskeletal pain, osteonecrosis of the jaw and ocular events. Moreover, pamidronate and zoledronate have been associated with renal complications (107). Association of bisphosphonates with atrial fibrillation and atypical fractures of the femoral diaphysis remains uncertain. There are a few case reports relating bisphosphonates to cutaneous reactions, oral ulcerations, hepatitis and oesophageal cancer. Generally, intravenous are more potent than oral bisphosphonates and the frequency and severity of some of the bisphosphonate-associated adverse events are dose and potency dependent. The risk of osteonecrosis of the jaw for high cumulative doses of zoledronate in patients with preceding dental defects has been widely reported (108). For example in myeloma patients, manufacturer-sponsored epidemiological studies reported the first estimates of the incidence of this toxic effect, ranging from 0.1% to 1.8%. By contrast, independent epidemiological efforts from clinicians and the International Myeloma Foundation reported incidence estimates between 5% and 10%. Between 2003 and 2005, warnings about the risks of bisphosphonate-associated osteonecrosis were disseminated by national regulatory agencies, the manufacturers of bisphosphonates, and the International Myeloma Foundation.

The second therapeutic option concerns denosumab, the fully humanized anti-RANKL antibody. It also induces failure of osteoclast activity and final inhibition of bone resorption, as observed with bisphosphonates. Three double-blind randomised trials including a total of about 6000 patients with bone metastases from solid tumours showed no tangible differences between denosumab and zoledronate in terms of mortality, disease progression, quality of life, or pain (109-111). Overall, toxicity was similar with denosumab and zoledronate in the 3 trials. Adverse effects which were more frequent with denosumab than with zoledronate included osteonecrosis of the jaw (1.8% versus 1.3%) and hypocalcaemia (9.3% versus 4.7%). However, renal failure was less frequent (2.6% versus 3.7%). Denosumab is administered subcutaneously and zoledronate by intravenous infusion. It is not known whether local and systemic reactions to administration are different. In practice, there is no tangible reason to choose denosumab rather than a bisphosphonate. **Therefore, both drugs should be proposed as adjuvant therapy to Ewing's sarcoma patients.**

7. Conclusion

Ewing's sarcoma patients are currently treated by adjuvant and neo-adjuvant poly-chemotherapy associated with surgery and radiotherapy in some cases. This therapeutic multi disciplinary approach only give rise to less than 25% overall survival for high risk patients, especially those with bone or bone marrow metastases. This is why new therapeutic options are urgently needed for high risk Ewing's sarcoma patients. The development of therapies which target the downstream signalling pathways activated by the fusion gene EWS-FLI1 are particularly attractive. However beside these targets, increasing number of data highlighted the role played by the tumour microenvironment which must be also considered as an interesting therapeutic option in the next future. Several pathways can thus be targeted such as hypoxia, angiogenesis or the bone cells themselves. In addition, interactions between tumour cells, tumour-derived humoral factors and the bone marrow in the bone niche have been shown to be essential for bone tumour initiation and promotion. Among the cells that constitute this particular microenvironment, osteoclast appear to be interesting targets as they participate to the vicious cycle that takes place during the progression of primary bone tumours. Osteoclast differentiation, activation and functions can be inhibited by two classes of molecules: bisphosphonates or antibodies directed against the cytokine RANKL. Respectively, two main drugs have been developed and are currently used in pathologies with high bone resorption activities: zoledronate and denosumab. Both drugs give interesting results in preclinical studies that encourage their transfer in clinic for Ewing's sarcoma patients.

8. Expert opinion

Targeting the cells present in the microenvironment, and the osteoclasts in particular represent indeed a new therapeutic option that must be deepened for patients with primary bone tumours such as Ewing's sarcoma in the future. The proof-of-concept has been demonstrated in preclinical studies using relevant animal models of Ewing's sarcoma, especially zoledronate which induced a strong inhibition of tumour progression in an orthotopic model of Ewing's sarcoma induced by injection of tumour cells in the medullar cavity of mouse tibia, which closely reproduce the clinical features observed in humans (81).

Because the drugs that target osteoclast differentiation and activation are already developed and used in other bone diseases where bone resorption is elevated (osteoporosis, bone metastases, multiple myeloma…), it is easy to propose their use as adjuvant therapy for patients with primary bone tumours including Ewing's sarcoma. Because Ewing's sarcoma can be considered as a rare disease, clinical trials must enrol large numbers of patients, and therefore must be proposed in large countries (United States or Europe). The bisphosphonate zoledronate (a member of the third generation of BP, the most potent inhibitor of bone resorption) is already proposed in the EWING2008 randomized phase III study in Germany and is planned to be also tested in the next future in the EWING2012 protocol in England, Italy and France. Although the anti-RANKL antibody denosumab represents the second most promising drug that may be used as adjuvant anti-bone resorption therapy in Ewing's sarcoma, no clinical trial plans to use it in Ewing's sarcoma patients in the next future. It must be noted that adverse side-effects have been already reported with the use of bisphosphonates (such as osteonecrosis of the jaw, renal deficiency or alteration of growth), denosumab may represent an interesting alternative.

Targeting the osteoclast activation represent a clever intuitive therapeutic option as these cells clearly participate to filling the vicious cycle between bone resorption and tumor progression. This concept has been already validated on other bone pathologies in which bone resorption is involved, leading to clear therapeutic benefit. It is therefore tempting to suggest that the same effect could be obtained in patients with primary bone tumours such as Ewing's sarcoma, as the same biological mechanisms have been described. The importance of tumour microenvironment is already accepted in other types of cancer, and the concept of its target must be more developed. It could also allow the patients to circumvent all the resistance mechanisms that are observed with the use of chemotherapy providing less adverse side effects than conventional drugs. One other possibility is the development of "bi-functional" molecules which could target the bone matrix in one hand (by the presence of a phosphonate residue), and target the tumour cells on the other hand (by the presence of an antitumour activity). This concept has been patented (WO/2009/083614 PCT/EP2009/050027), Nantes University/Nantes Hospital/INSERM/CNRS, "Hydroxy-bisphosphonic acid derivatives as vector for targeting bone tissue") and is currently under development for primary bone tumours in preclinical studies (112). This

family of molecules can provide better targeting of anti-tumour activity in bone site, with limiting systemic side effects.

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Legends to Figures and tables

Table 1: Ewing's sarcoma: Classification of Histological response to pre-operative chemotherapy according to Salzer-Kuntschick grading

Table 2: Ewing's sarcoma: Classification of surgical procedures according to Enneking grading.

Figure 1: Classical histology of Ewing's sarcoma tumors (Hematoxylin & Eosin staining) characterized by small round cells (magnitude: x 40).

Figure 2: Current EuroEWING99 protocol proposed for Ewing's sarcoma patients in Europe.
VIDE: Vincristine-Ifosfamide-Doxorubicin – Etoposide; VAI: Vincristine – Actinomycin D – Ifosfamide; VAC: Vincristin – Actinomycin D – Cyclophosphamide; R1: localized disease ; R2: pulmonary metastases ; R3: bone and/or bone marrow metastases

Figure 3: Characteristic X-ray film radiographs (A) and MRI (B) of Ewing's sarcoma in the pelvis with associated bone lesions (arrows).

Figure 4: The vicious cycle between tumour cell proliferation and bone cells (osteoclasts and osteoblasts). Tumour cells produce osteoclast activating factors such as Interleukin-1 (IL-1), IL-6, Tumour Necrosis factor (TNF-α), TNF-β, Transforming growth factor-beta (TGF-β), Parathyroid Hormone-related peptide (PTH-rP) which mediate Receptor Activator of NF-kB Ligand (RANKL) production (directly or via osteoblasts). RANKL binds to its receptor RANK present at the surface of osteoclasts or precursors, inducing their differentiation and activation in mature cells able to degrade bone. When they resorb bone, osteoclasts allow the release of growth factors such as TGF-β or Insulin-like growth factor (IGF)-1 stored in the bone matrix that in turn activate tumour cell proliferation. Osteoprotegerin (OPG) can block this cycle by inhibiting the RANKL-RANK interaction.

Figure 5: Immuno-histochemical analysis of RANKL production in human biopsy of Ewing's sarcoma patient (Magnitude x40 and x100).

Figure 1

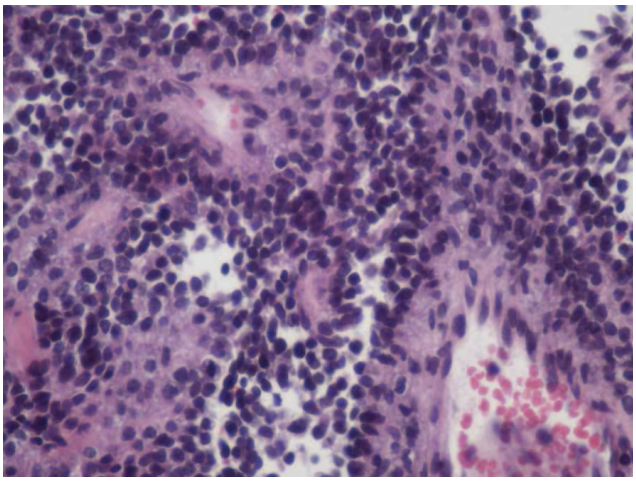
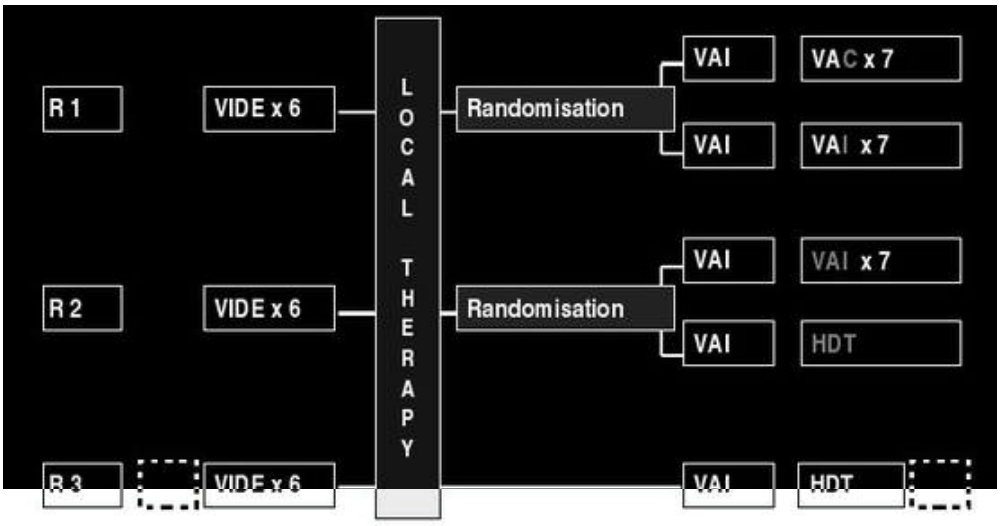


Figure 2


R 1
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Randomisation
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VAC x 7
VAI
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R 3
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Figure 3

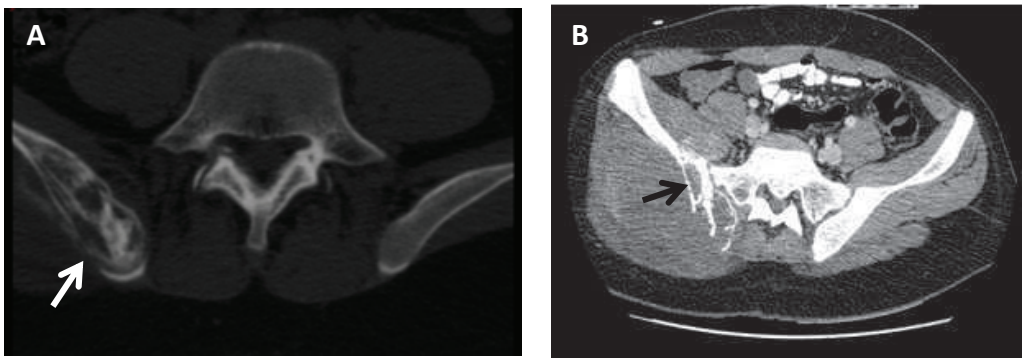

Figure 4

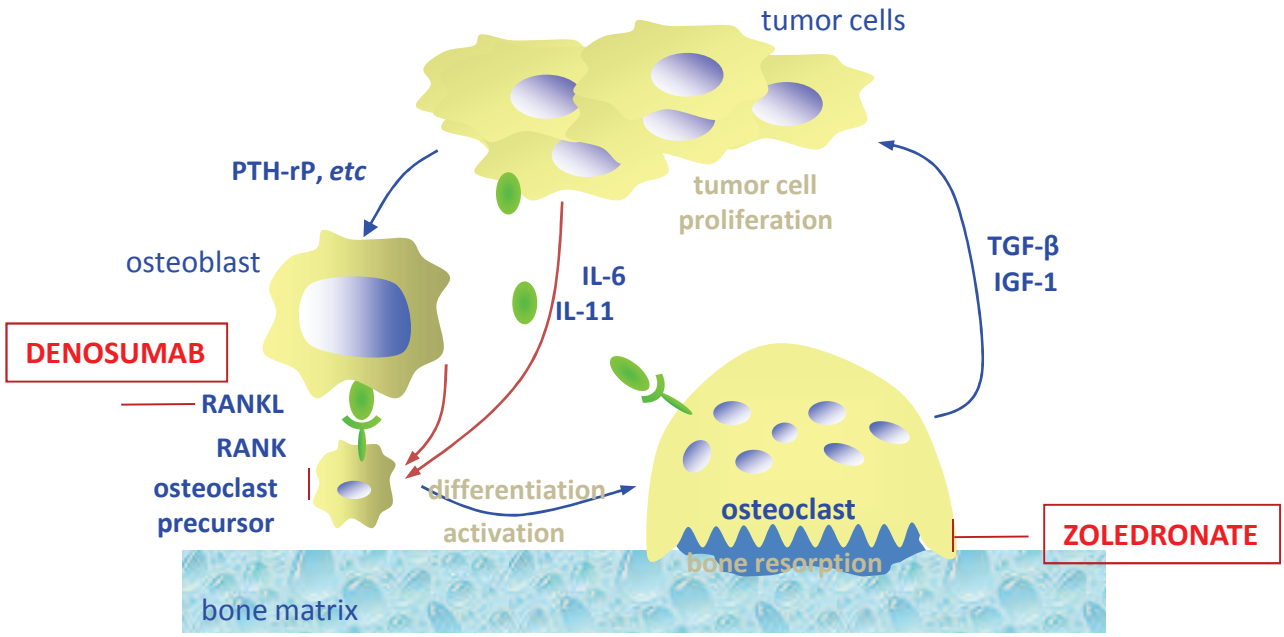
tumor cells
PTH-rP, *etc*
tumor cell
proliferation
TGF-β
IGF-1
osteoblast
IL-6
IL-11
DENOSUMAB
RANKL
RANK
osteoclast
precursor
differentiation
activation
osteoclast
bone resorption
ZOLEDRONATE
bone matrix

Figure 5

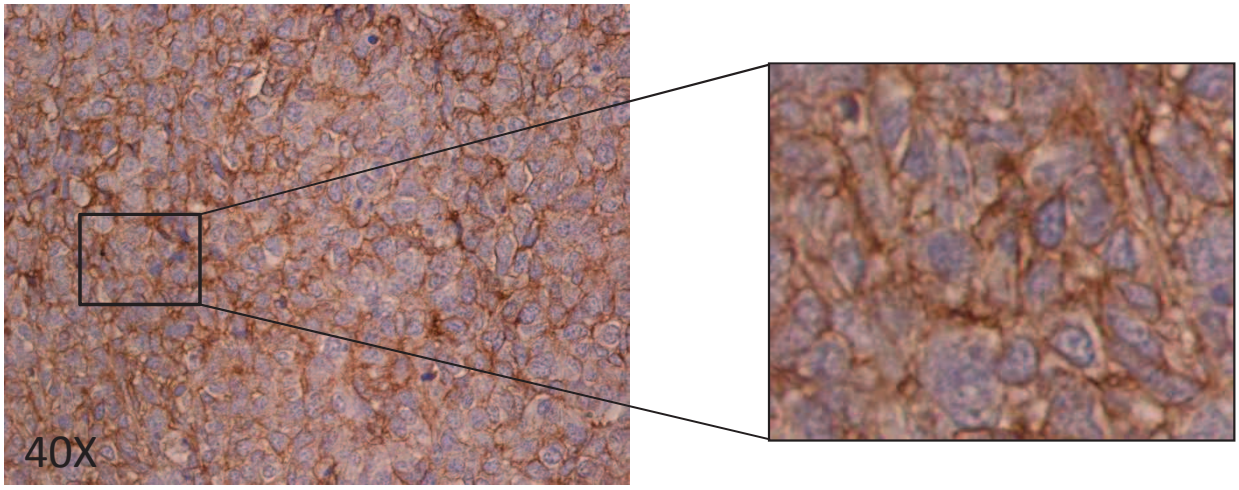

Table 1

Enneking grading	Definition
Radical/wide	tumour completely removed; tumour remained intact under surgery; macroscopically and microscopically completely surrounded by intact, healthy tissue or « capsule »; bioptic channel removed en bloc with the surgical material with sufficient safety margin.
Marginal	tumor completely removed; tumour remained intact under surgery; possibly microscopic evidence of tumor tissue t the surgical margins; no evidence of tumour residues at tumour site.
Intralesional	tumour incompletely removed or opened under surgery or evidence of tumour tissue at the surgical margins, macroscopic evidence of tumour residue.;

Table 2

Grading	Definition
Grade 1	no evidence of viable tumour cells
Grade 2	single viable tumour cell island (<0.5cm)
Grade 3	< 10% viable tumour cells
Grade 4	10-50% viable tumour cells
Grade 5	> 50% viable tumour cells
Grade 6	no effect of chemotherapy