

Potential role of epidermal growth factor receptor pathway and connexin 43 communication channels in giant cell tumour of bone

Doctoral theses

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1. Introduction

1. 1. Giant cell tumour of bone

The giant cell tumor of bone (GCTB) is classified by World Health Organisation (WHO) as a locally aggressive osteolytic tumour of intermediate malignancy, representing 5% of primary bone tumours. GCTB usually originates from the epiphyseal area of long bones, but in skeletally immature patients it may occur also in the metaphyseal region. Based on literature data, 80% of GCTB cases have a benign course, 12-27% of cases may recur locally, less than 1% of GCTB cases may display malignant transformation, and in 1-4% it may form benign lung implants, which may also be considered as metastases.

GCTB is made up of three main cell types. The mononuclear cells can be divided to CD68-/CD163-positive oval monocyte/macrophage lineage cells and CD68-negative spindle shaped neoplastic stromal cell population. The latter is the proliferative component, which can be positive for α -smooth muscle actin (α -SMA). GCTB stromal cells show osteoblastic phenotype, and they produce receptor activator of nuclear factor-kappa B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). The third cell type is the CD68-positive multinuclear osteoclast type giant cells of monocyte/macrophage origin, which are responsible for osteolysis. Osteoclasts formation is mainly driven by RANKL and M-CSF canonical pathway, but alternative non-canonical pathways involving hypoxia and other cytokines and growth factors have also been revealed.

Cytogenetic alterations as telomeric associations (TAS) or telomeric fusions were frequent (85%) in GCTB neoplastic stromal cells. TAS as clonal phenomenon may be manifested as an intermediate step or precursor lesions towards further structural aberration. In addition, a structural alterations numerical chromosome changes were described mostly like polysomies or balanced individual cell aneusomies.

GCTB is mostly treated surgically using curettage followed by local phenol adjuvant treatment. The cavity resulted from the extended curettage is filled with polymethyl methacrylate (PMMA). Combined surgical and adjuvant treatments can result in reduced recurrence rates. Radiation therapy may be used in cases where the tumor is hard to reach or cannot be removed surgically. Bisphosphonates may also be used to inhibit malignant and benign bone resorption. Denosumab is a human monoclonal antibody (IgG₂), which can bound to soluble and membrane bound RANKL produced by osteoblasts, and bone marrow stromal cells for inhibiting osteoclastogenesis.

1. 2. Epidermal growth factor receptor (EGFR)

The ErbB receptor family of type I receptor tyrosine kinases (RTKs) plays important role in development of a multicellular organism, because the receptor signalling pathways regulates cells proliferation, survival, differentiation and migration. EGFR is a 170 kDa molecular weight trans-membrane glycoprotein comprised of three domains: extracellular ligand binding domain, trans-membrane and intracellular domain, which is composed of conserved catalytic activity protein tyrosine kinase subdomain and C-terminal regulation region. The ligand bound to the receptor leads to conformational change and receptor dimerization, which results in auto- and trans-phosphorylation of tyrosine residues on tyrosine kinase subdomain on C-terminal regulation region. Adaptor proteins and cytoplasmic tyrosine kinases bound to the phosphorylated tyrosine residue, activates downstream signalling including mitogen activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3-K), phospholipase C gamma (PLC γ) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathways.

Bone formation and bone resorption are regulated by large number of hormone, growth and transcription factors. EGFR mediated pathways take part in regulating anabolic bone metabolism.

1. 3. Connexins

Direct cell-cell communication mediated by coupling of cell membrane hemichannels (gap junction–GJ) composed of 6 connexin (Cx) proteins, exclude extracellular space for transporting regulatory molecules between adjacent cells. Connexin channels permit the transfer of ions (K⁺, Ca²⁺) and small molecules of <1.8 kDa such as metabolites (glucose), and second messengers (IP3, cAMP, cGMP) between coupled cells. GJ cannels plays important roles in regulating tissue homeostasis, embryogenesis and basic physiological functions including cell growth, differentiation and in mediating the direct propagation of action potentials within excitable cell networks. Connexin channels also transport nutrients in avascular tissues such as the bone.

Cx isotypes are composed of four hydrophobic trans-membrane domains (M1-M4), two extracellular hydrophilic loops (E1 and E2), an intracellular hydrophilic cytoplasmic loop (CL), a C-terminal and an N-terminal domain. Six Cx polypeptides oligomerize and forms hemichannels, also called connexon. Two connexons in adjacent cells can join to form a GJ

channel, where the gap between cells is reduced to 2-3 nm. Hundreds and thousands of these channels arrange into organized plaques in cell membranes, and work either as GJ channels or hemichannels.

GJ channels formed by Cx43 protein plays an important role in bone development, osteoblast proliferation and regulation of differentiation. They support osteocyte adaptation to mechanical strain and soluble growth factors induced stimuli and supports the survival of osteocytes through apoptosis inhibition. Missense mutations of the GJA1 gene encoding the Cx43 protein cause skeletal malformations called as oculodentodigital dysplasia (ODDD). In mice, induced ODDD-like mutations resulted in increased osteolysis.

2. AIMS

In GCTB we examined:

1. The relationship between EGFR protein expression and tumour recurrence and aggressiveness.
2. Potential EGFR gene amplification and tyrosine kinase domain mutations in the background of elevated EGFR protein expression.
3. The effect of EGF administration on GCTB stromal cells proliferation, differentiation, osteoclastogenesis and osteoclast activation.
4. Cx43 protein expression in relation to tumor aggressiveness and progression free survival (PFS).
5. In cultured GCTB stromal cells the expression of Cx43 both at mRNA and protein level, the subcellular localisation and function of Cx43 channels, compared to control cells including bone marrow stromal cells and fibroblast cell line.

3. MATERIALS AND METHODS

3. 1. Patient cohort

In a co-operation within EuroBonet and EU FP6 project for studying bone tumors, we tested samples formalin fixed paraffin embedded GCTB of 260 patients (EGFR study: 118 SE and 149 IOR, and in Cx43 study: 123 IOR), collected between 1977-2005 at Department of Orthopaedics, Semmelweis University (SE, Budapest, Hungary) and 1994-2005 at Institute of Orthopaedics Rizzoli (IOR, Bologna, Italy).

3. 2. Tissue microarray (TMA)

Duplicate tissue cores were collected from archived tissue samples into tissue microarrays (TMA). The included 4 x 70 samples (IOR) and 3 x 80 samples (SE) into TMA blocks, which were cut into 3-4 μm thick sections.

3. 3. Immunohisto- and immunocytochemistry

TMA sections were immunostained for EGFR, pEGFR (pY1068 and pY1173), EGF, transforming growth factor alpha ($\text{TGF}\alpha$), alpha-smooth muscle actin (α -SMA), CD11c, CD163 and tartrate-resistant acid phosphatase (TRAP), while cell cultures TRAP, vitronectin receptor (VNR), vimentin and Cx43 proteins were detected using immunohisto- and immunocytochemistry. Visualization of the reactions was made either using horse radish peroxidase (HRP) conjugated anti-rabbit/mouse IgG polymer (Novolink polymer) and alkaline phosphatase (ALP) conjugated anti-mouse IgG with 3,3'-diaminobenzidine (DAB) or Permanent Red Substrate-Chromogen detection system, respectively. For immunofluorescence Alexa546 (red) or Alexa488 (green) fluorochrome conjugated anti-mouse IgG or anti-rabbit IgG was used. Nuclei were stained either with hematoxylin or with 4',6-diamidino-2-phenylindole (DAPI).

3. 4. Cell culturing and *in vitro* treatments

Stromal cells were isolated from fresh GCTB tissues and normal bone marrow and were cultured. Human peripheral blood mononuclear cells (PBMC) were isolated from venous blood with Ficoll density gradient centrifugation. Human Dermal Fibroblasts-Adult (HDFa), mouse

osteoblastic (2T3) and human non-small cell lung cancer (NSCLC) (H1650, H1975 és H358) cell lines were used as a control.

In the osteoclast differentiation study using dentine slices and glass coverslips cultured PBMCs were treated with either M-CSF, RANKL, M-CSF + RANKL, EGF + M-CSF, EGF + RANKL and EGF + M-CSF + RANKL. After toluidine blue staining of dentin slice resorption areas were quantified.

After the examination of GCTB stromal cells proliferation, cells were incubated with either 0, 10, 25, 50 or 100 ng/ml final concentration of EGF, than the cells were incubated with CellTiter-Blue® Reagent (Promega). In metabolic active cells resazurin was reduced to resofurin, which fluorescence intensity was proportional with the live cells number.

For GCTB stromal cell differentiation the ALP enzyme activity of osteoblasts was tested after EGF treatment using AS-MX phosphate substrate solution and Fast Violet B chromogen.

3. 5. Fluorescent in situ hybridization (FISH)

The copy number of EGFR gene on TMA sections was determined with centromere-, and locus-specific EGFR/CEP 7 dual probe (ZytoVision), the neoplastic nature of GCTB stromal cell culture was confirmed based on numerical and structural alterations determined using chromosomes 3, 4, 6 and X specific alpha satellite, or 11p subtelomeric and chromosome 11 specific alpha satellite probes.

3. 6. EGFR tyrosine kinase domain mutation analysis

After DNA extraction from the TMA sections, exons 19 and 21 of the EGFR gene were amplified using nested PCR. Obtained PCR products were reamplified using mutant-enriched PCR, which was based on intermittent restriction digestion to eliminate wild-type genes selectively, thus enriching the genes with exon 19 deletion or exon 21 point mutation. After purification of PCR product, direct sequencing of exons 19 and 21 of the EGFR gene was performed with the BigDye® Terminator v3.1 Sequencing Kit, which products were detected with automated fluorescent capillary electrophoresis (ABI PRISM® 310 Genetic Analyzer, Thermo Fisher Scientific) system.

3. 7. Gene expression

RNA was isolated (RNeasy[®] Mini Kit, Qiagen) and reverse transcribed. Real-time PCR was used to investigate the gene expression of Cx43, RANKL and OPG in GCTB stromal cell cultures. Gene expression was normalized to β -actin.

3. 8. Western blot

Cx43 protein expression was examined in cultured GCTB stromal cells, bone marrow stromal cells and HDFa cell line using polyacrylamide gel electrophoresis followed by protein blotting on PVDF membrane. For secondary antibody HRP conjugated anti-rabbit IgG was used and the reaction was developed with chemiluminescence (ECL, Western Blotting Substrate, Pierce) detection system. β -actin was used to confirm equal protein loading.

3. 9. Flow cytometry

Functional cell-cell communication analysis was performed with a double dye labelling assay. From the non-fluorescent calcein acetoxymethyl ester (calcein AM) hydrophilic fluorescing calcein is generated in live cells by esterases, which can pass through connexin channels between adjacent cells. 1,1'-dioctadecyl-3,3,3'-tetra-methylin-dodicarbocyanine (DiI) is an lipophilic red fluorescent dye bond into cell membranes indicating calcein labelled donor cells. Double labelled cells were mixed with unlabelled recipient cells at a ratio of 1:9, and co-cultured for 12 hours and then the ratio of single calcein-positive cells, as a measure of metabolic dye coupling, was determined with flow cytometer.

3. 10. Digital microscopy, scoring and image analysis

Immunostained TMA sections were digitalized with the Panoramic Scan (3DHISTECH Ltd.) digital microscope. EGFR reaction intensity was examined using a 12-scale system, consolidated for weak (1-4), moderate (5-8) and strong (9-12) staining. Cx43 reaction frequency of positive mononuclear cells were examined using an 8-scale system: 0: <3%; 1+: 3-5%; 2+: 6-10%; 3+: 11-20%; 4+: 21-30%; 5+: 31-40%; 6+: 41-50%; 7+: 51-60%; 8+: >60% positive tumor cell. FISH signals were also studied with Panoramic Viewer software and immunofluorescence signals were examined with HistoQuant module software.

3. 11. Statistical analysis

Statistical analyses were made using the SPSS 15.0 and Statistica 8.0 softwares. The correlation between EGFR expression and GCTB disease progression was tested with both the Pearsons' Chi-square (χ^2) test and Spearmans' rank correlation analysis. The relationship between Cx43 expression and clinicoradiological stage and progression were analysed using the non-parametric Johnkeer-Terpstra test for multiple variables followed by the Mann-Whitney U for pairwise comparisons using the Bonferroni or Holm-Hochberg correction. Survival curves were displayed in Kaplan-Meier plots, where log-rank test and Cox regression analysis was used for testing statistical significance between groups. Univariate Cox regression analysis was used for testing correlations between Cx43 expression and progression free survival (PFS), while multivariate Cox regression analysis was used for testing correlations between Cx43 expression and gender, age, grade, localization and first treatment. In cell cultures, immunoreactions tested with image analysis, mRNA and protein levels measured with RT-PCR and Western blot respectively were tested using the independent samples Student t-test. The value $p < 0,05$ was considered as statistically significant.

4. RESULTS

4. 1. EGFR signalling in giant cell tumour of bone

4. 1. 1. EGFR expression in giant cell tumour of bone

EGFR protein expression has proved by several EGFR specific antibodies. Using the antibody clone 3C6 resulted in moderated to strong membrane immunoreactivity and a weak cytoplasmic reaction for EGFR in spindle shaped mononuclear cells in the osteoclast rich regions in 76% (176 of 235) of GCTB cases. Significantly elevated EGFR expression, using antibody clone 3C6, was seen in recurrences (71 of 92; 77%; $p < 0,002$) and malignant (22 of 26; 86%; $p < 0,001$) cases compared to non-recurrent cases.

More than 20% of spindle shaped mononuclear stromal cells showed phosphorylated (activated) tyrosine kinase domain positivity in 72% of EGFR (3C6) positive GCTB cases. Phosphorylated EGFR reaction indicating activation of the tyrosine kinase domain showed positive correlation with EGFR expression detected with antibody clone 3C6 ($p < 0,05$).

4. 1. 2. Clinicopathological correlation of EGFR expression

EGFR-positive cases were significantly more frequent in clinico-radiologically aggressive (31 of 43; 72%) than in latent (27 of 54; 50%) cases ($p = 0,034$). In some aggressive cases the EGFR-positive stromal cells invaded adjacent skeletal muscle.

4. 1. 3. Determination of EGFR expressed cell type

EGFR immunoreaction was frequently detected in α -SMA-positive neoplastic stromal cells. CD163-positive monocytes/macrophages and their progeny, differentiated osteoclasts rarely showed any EGFR reaction.

4. 1. 4. Determination of EGFR gene status

In EGFR-positive GCTB cases 5-20% of mononuclear cells showed numeric alterations in chromosome 7 manifested as tri- or tetrasomy, however EGFR gene amplification was not detected.

4. 1. 5. EGFR gene tyrosine kinase domain 19 and 21 exons mutation analysis

Neither exon 19 deletion nor exon 21 point mutations of the tyrosin kinase domain of EGFR gene were detected with mutation-enriched RT-PCR.

4. 1. 6. Production of EGFR ligands in GCTB

EGF immune reaction was detected primarily in monocyte/macrophage and endothelial cells. Stromal cells were negative for both EGF and TGF α ligands. TGF α , localized around cell nuclei in osteoclasts and macrophages and only rarely and weakly detected in stromal cells.

4. 1. 7. TRAP-positive osteoclasts and EGFR expression in GCTB

The number of TRAP-positive osteoclasts was significantly higher in EGFR-positive (735 ± 237) than in EGFR-negative cases (581 ± 237) ($p = 0,02$). Strong EGFR protein expression was associated with larger number and smaller size of TRAP-positive osteoclasts, than in EGFR-negative cases. The average size of TRAP-positive osteoclasts was significantly ($>18\%$) smaller in EGFR-positive than in EGFR-negative cases ($p < 0,05$).

4. 1. 8. EGF treatment and osteoclastogenesis

The treatment of PBMCs with the combination of EGF and M-CSF resulted in similar number of VNR- and TRAP-positive multinucleated osteoclasts, to that gained when M-CSF and RANKL was combined. However, as opposed to M-CSF and RANKL, the EGF and M-CSF combination did not elevate osteoclasts activity. The number of VNR- and TRAP-positive multinucleated osteoclasts were significantly higher (VNR $p = 0,024$ and TRAP $p < 0,001$) after EGF and M-CSF treatment, than after M-CSF treatment alone.

4. 1. 9. EGF treatment and GCTB stromal cells proliferation/differentiation

Cultured GCTB stromal cells treated with 100 ng/ml EGF showed increased proliferation at third and ninth day ($p < 0,05$). ALP activity was measured as an osteoblast differentiation marker. Stromal cells ALP level did not change significantly with increasing EGF concentration.

4. 2. Cx43 expression and direct cell-cell communication in GCTB

4. 2. 1. Clinicopathological correlations of Cx43 expression in GCTB

Cx43 plaques were most expressed in the CD163-negative neoplastic stromal cells ($81,7 \pm 12,56\%$). Furthermore, α -SMA-positive GCTB stromal cells were linked to significantly less Cx43 ($32,6 \pm 13,4\%$), than α -SMA-negative cells ($p = 0,017$).

Cx43 expression was significantly higher in the tumor surrounding reactive stroma, than in the osteoclasts rich tumor nests. This was related both to the percentage of Cx43-positive area ($p < 0,001$), and the number of Cx43 plaques ($p < 0,016$). There was no significant link between Cx43 expression and the frequency of GCTB recurrences ($p = 0,173$). However, Cx43 expression showed an inverse link with the clinico-radiological tumor stage. There were significantly more GCTB cases showing low Cx43 level in the aggressive tumors than in latent ($p = 0,002$, Bonferroni correction: $p < 0,0167$) or in the active tumors ($p = 0,018$, Holm-Hochberg correction: $p < 0,025$).

Scored on a 0 to 8 scale the higher Cx43 protein level ($n = 123$) was significantly associated to better PFS.

4. 2. 2. Neoplastic nature of GCTB stromal cells tested with FISH

Isolated and cultured GCTB stromal cells displayed a wide range of polysomies for chromosomes 3, 4, 6 and X. We observed individual cell aneusomies, chromosome 4 trisomy and chromosome 11 tetrasomy and the subtelomeric loss of chromosome 11.

4. 2. 3. Connexin43 protein expression in GCTB stromal cells

In cultured GCTB stromal cells, most Cx43 protein was localized to the endoplasmic reticulum (ER)-Golgi region instead to be transported to the cell membranes. Cell membrane bound Cx43 was significantly higher in HDFa cell line ($p < 0,01$) and in bone marrow stromal cells ($p < 0,05$) than in GCTB stromal cells.

4. 2. 4. Cx43, RANKL and OPG mRNA expression in GCTB stromal cells

Quantitative RT-PCR displayed significantly reduced ($p < 0,01$) Cx43 mRNA expression in GCTB stromal cell culture, compared to either of control cells. Osteoclastogenic RANKL mRNA level was not detected in bone marrow stromal cells and it was significantly

lower in the HDFa cell line ($p < 0,01$), than in GCTB stromal cells. In line with this, mRNA levels of the osteoclastogenesis inhibitor OPG were significantly higher in bone marrow stromal cells and HDFa cell line ($p < 0,001$) than in GCTB stromal cells.

4. 2. 5. Cx43 phosphorylation analysis in GCTB stromal cells using Western blot

Cx43 protein expression in GCTB stromal cells was also significantly lower than in control bone marrow stromal cells or in HDFa cell line ($p < 0,05$). Cx43 immunoreaction demonstrated two alkaline phosphatase sensitive extra bands (P1 and P2) both in HDFa cell line and in primary bone marrow stromal cell isolates, which were missing from primary GCTB stromal cells.

4. 2. 6. Testing of metabolic coupling through gap junction channels using dye transfer assay

In GCTB stromal cells dye transfer was significantly reduced compared to control cells. In GCTB stromal cells the average number of single calcein fluorescing cells was 0,98%, which was significantly lower ($p < 0,001$) than in control HDFa cell line (7,13%) or in bone marrow stromal cells (4,6 %).

5. CONCLUSIONS AND NOVEL OBSERVATIONS

1. EGFR protein was mostly associated with neoplastic stromal cells. Activation of EGFR was confirmed by detection of the C-terminal phosphorylated tyrosine domains (pY1068 and pY1173), which was present in 72% of EGFR-positive cases.
2. EGFR levels showed positive statistical correlation with GCTB progression (recurrence) and clinico-radiological aggressiveness.
3. Neither EGFR gene amplification nor EGFR gene tyrosine kinase domain mutations were detected in background of elevated EGFR protein. This suggest the epigenetic regulation of EGFR signalling in GCTB.
4. *In vitro*, EGF treatment stimulated GCTB stromal cells proliferation, which may supports osteoclastogenesis indirectly without activating osteoclasts functions.
5. Cx43 protein expression in neoplastic stromal cells showed negative statistical correlation with GCTB clinico-radiological aggressiveness and positive correlation with the PFS of the patients.
6. In GCTB stromal cell culture Cx43 protein was stack in the ER-Golgi network instead of transported into the cell membranes and showed defective phosphorylation. As a result, significantly reduced metabolic cell-cell communication was seen in GCTB neoplastic stromal cells.
7. In GCTB stromal cell culture reduced Cx43 and OPG mRNA levels were associated with elevated RANKL mRNA level, compared to reactive control cells. Knowing that Cx43 mutations lead to elevated osteolysis our findings suggest a role of deregulated Cx43 channels and cell-cell communication in pathological osteoclastogenesis.

6. PUBLICATIONS

6. 1. Publications in the subject of the dissertation

1. **Balla P**, Maros ME, Barna G, Antal I, Papp G, Sapi Z, Athanasou NA, Benassi MS, Picci P, Krenacs T (2015) Prognostic impact of reduced connexin43 expression and gap junction coupling of neoplastic stromal cells in giant cell tumor of bone. *PloS One* 5: e0125316.
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6. 2. Publications in different subject

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