Investigation of effects of tyrosine kinase inhibitor imatinib and nilotinib widely used for treatment of oncohematological diseases on osteoblasts gene expressions

Ph.D Thesis

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1. Introduction

Creating the present study was inspired by the observations of the studies presented in the international literature that were performed with tyrosine kinase inhibitors. Numerous clinical observations have confirmed that breakpoint cluster region-abelson fusion oncoprotein tyrosine kinase inhibitors (for example imatinib) used in leukemia treatment alter bone physiology in a complex and unclear manner (for example function of bone cell, bone turnover, bone mineral density). During the initial research a lot of scientific studies tell about the fact that significant increase in bone mass has been observed because of the effect of tyrosine kinase inhibitors. However at that time we did not know much about the mechanism of action of the processes and changes of function of signaling pathways.

As the tyrosine kinase inhibitors are widely used drugs for the treatment of certain oncohematological diseases, and the treatments may continue for decades or the rest of the patients' lives, therefore it is important to know more about the background of these molecular mechanisms.

2. Our Purpose

In this study the changes in the signaling pathways and gene expression of osteoblasts have been examined through two well-known compounds, tyrosine kinase inhibitors because of the significant effects of the tyrosine kinase inhibitors on the bone metabolism presented in the scientific literature,

The purpose of our research is to observe the effects of imatinib and the more selective BCR-ABL inhibitor molecule nilotinib on gene expression patterns of murine osteoblast in in vitro system.

Tasks to obtain:

- Studying and more thorough understanding of the known signaling pathways in connection with bone diseases. As the important role of Wnt signaling pathway in bone metabolism was previously also confirmed as the result of the cooperation with Péter Horváth, so we take particular attention to the examination of the role of this pathway while performing our research with tyrosine kinase inhibitors.

- After defining the adequate concentration and incubation time, the examination the effect of imatinib and nilotinib on gene expression of MC3T3-E1 murine osteoblasts by whole transcriptome analysis based on the next generation sequencing.
- After the statistical analysis of the genes identified by sequencing we realise signaling pathways, top upstream molecules and top molecules using the signaling pathway analysis with both drugs.
- To examine how the identified changes confirm the previous clinical observations.
- To examine considering the results if the effect of imatinib and nilotinib on gene expression is the same.

3. Methods

The experiments were performed on MC3T3-E1 cultured murine osteoblasts. In the *in vitro* system, the following three sample groups were examined: imatinib-treated, nilotinib-treated and untreated (control) osteoblast cell cultures. In any case, we performed 3 parallel

measurements, and in the end the parallel biological samples were pooled.

After the cell culturing, the adequate incubation time and drug concentration were defined using a cell viability assay. Different imatinib (Glivec®/Gleevec®, STI571, CGP 57148B; Novartis, Basel Switzerland) and nilotinib (Tasigna®; Novartis) concentrations (30 nM – 20 μM) were administered to osteoblasts for various incubation times (1-6 days) in 96-well plates. As the tyrosine kinase inhibitors are widely used drugs for the treatment of certain oncohematological diseases, and the treatments may continue for decades or the rest of the patients' lives, therefore during our experiments we tried to use the longest treatment, which was available in in vitro system. Thus the presented results reflect the extended secondary expression changes.

In parallel the measurements of cell viability, the bone specific gene expression effects of different drug concentrations and incubations time on osteoblasts were also investigated by relative quantification real time polymerase chain reaction (RT-PCR) method.

After the definition of optimal incubation time and drug concentration, RNA isolation was performed from the

treated and untreated osteoblasts. The whole transcriptome analysis (WTA) of the purified, DNA-free total RNA molecules were performed by SOLiD next generation 50+20 bp reads paired-end technologies.

In biological samples coding and noncoding RNA molecules can be defined relatively cheaply and quickly by whole transcriptome analysis. Therefore WTA can quantitatively detect the gene expression changes. The precise evaluation of huge amount of data can be performed by modern bioinformatics software.

During the WTA the level of all mRNA molecules expressed in cell can be defined quantitatively and genes expressed in altered manner in bone cells, because of the effect of tyrosine kinase inhibitors, can be also defined. After defining the statistically significant altered genes, canonical pathway analysis can be performed.

The signaling pathways, top upstream regulators and top molecules which were defined by canonical pathway analysis during our experiments, support the better understanding of effect of imatinib and nilotinib on osteoblast gene expression. During the evaluation special attention was paid to the interpretation and explanation of

the suspected bone metabolism modifying effects of imatinib and nilotinib.

4. Results

Results of imatinib and nilotinib concentration and incubation time optimizer in vitro experiments

Based on the results of cell viability measurements and relative quantification RT-PCR experiments, the determined optimal settings are: 1 μM drug concentration and 6 days of incubation time. After pooling of parallel biological samples RNA isolations were performed from the treated and untreated osteoblasts for WTA.

Identified RNA molecules in osteoblasts:

We identified 16.383 annotated RNA in the imatinib-treated group, 16.951 ones in the nilotinib-treated group, and 17.290 ones in the untreated control group. After that we identified the genes showing significantly changed mRNA expression levels, in the case of both drugs.

Identified top canonical pathways:

Genes showing significantly altered expression rates in response to the two drugs compared with control were evaluated by IPA 7.6 canonical pathways analysis software. Six top canonical pathways were identified in the imatinib-treated osteoblast cells (Reelin signaling, fatty acid activation pathway, GABA receptor signaling, sertoli cell - sertoli cell junction signaling, γ-linolente biosynthesis II, Serotonin receptor signaling). Five top canonical pathways were identified in the transcriptome of the nilotinib-treated group (eukaryotic Initiation Factor 2 (EIF2) signaling pathway, embryonic stem cell differentiation into cardiac lineages, transcriptional regulatory network in embryonic stem cells and role of Oct4 in mammalian embryonic stem cell pluripotency pathways, GABA receptor signaling pathway).

At the imatinib-treated and nilotinib-treated groups the identified pathways were different with one exception (GABA receptor signaling), in addition the expected role of Wnt signaling pathway did not improve in any groups.

Identified top upstream regulators:

Five upstream regulators were identified in the imatinib group, namely: FREM2, GLDN, GRIP1, NRCAM, and VLDLR. From these, the FREM2, GRIP1 and VLDLR can take part in bone physiological process in indirect manner.

In the nilotinib-treated osteoblast culture, five top upstream regulators were also found: ACVR1B, ACVR1C, FAAH, MARCH7 and RAD23B. In case of nilotinib treatment, there are known data in connection with bone metabolism on all five upstream regulators.

Identified top molecules:

The most sensitive genes were determined after imatinib and nilotinib treatment. The top molecules were selected based on logarithmic fold change (logFC) values, which describe the rate of expression changes in the treated groups compared with the untreated control group. In the case of both drugs the top molecules were defined. In the imatinib-treated group ten top molecules were identified: AQP9, CSMD1, Eda, GDF10, HYDIN, MYO3B, NYAP2, RELN, SLITRK5 and STXBP5L. In the nilotinib-treated group nine top molecules were identified:

DKK4, EDNRB, GABRB1, KLHL41, NANOG, RPL17, RPL39, RPS23, and ZFP184. Due to imatinib application the STXBP5L gene showed the strongest upregulation (21-fold). Furthermore nilotinib usage the RPS23 gene had the strongest 11-fold increase in expression.

5. Conclusions

Numerous clinical observations have confirmed that breakpoint cluster region-abelson fusion oncoprotein tyrosine kinase inhibitors used in leukemia treatment alter bone physiology in a complex manner. Results of our experiments also improve the effect of tyrosine kinase inhibitors on osteoblasts.

To the best of our knowledge, this is the first study to observe the complete mRNA pattern of osteoblasts by whole transcriptome analysis. Thus, it was possible to demonstrate the top canonical pathways and upstream regulators that were affected in osteoblast cells by these compounds. Previous studies have reported expression data in response to tyrosine kinase inhibitors only of strictly bone-related genes (RANKL, OPG, bone sialoprotein, osteocalcin, osterix, BMP2 and RUNX2).

Nevertheless, complex transcription pattern analysis in connection with bone metabolism have not been performed so far.

During our experiments, in case of both drugs, genes showing significantly changed mRNA expression levels, top signaling pathways, upstream regulators and top molecules were defined. We paid particular attention to the interpretation of hypothesized bone metabolism modification effects of these drugs. Based on our results, the effects of the two investigated tyrosine kinase inhibitor on bone metabolism do not happen through the well-known signaling pathways connection with bone metabolism (for example Wnt signaling pathway which was also identified as the result of the cooperation with Péter Horváth). Our data have indicated the potential role of a number of genes and signaling cascades that may regulate the function of osteoblasts. The knowledge which we obtained by doing our work supports more detailed understanding of mechanism of action of the two compounds. This knowledge may contribute to identify novel targets for the treatment of metabolic bone diseases in the future. Considering the newer research results (including our own results) we can declare that the widely

used tyrosine kinase inhibitors for the treatment of oncohematological diseases have no unequivocal positive effects on bone metabolism. In the effect of investigated tyrosine kinase inhibitors on bone the pathways strictly connected to the bone metabolism do not play any role. So the Wnt pathway we supposed do not play any role either. In addition the effects of imatinib and nilotinib on osteoblast function depend on the concentration of the utilized tyrosine kinase inhibitors, the maturation stage of the osteoblasts and the distribution of various tyrosine kinase inhibitor-targeted receptors on cells.

Our results indicate different effects of imatinib and nilotinib on osteoblast function, and the effect of imatinib is stronger. Among the significantly altered genes in the two treatment groups, there were only three common genes (Zfp184, Gm11225 and AI593442). Furthermore mostly different signaling pathways, upstream regulators and top molecules were identified in the two treated groups.

Based on our experiments the effects of imatinib and nilotinib are different. The explanation of this phenomenon may be the fact that the investigated two compounds have different chemical structures and

different kinase profile. In addition imatinib can block more tyrosine kinase receptors, while the more selective nilotinib cannot block or only hardly blocks the majority of tyrosine kinase receptors.

During our experiments the completed treatments with tyrosine kinase inhibitors on cultured osteoblasts support the better understanding of mechanism of action of widely used imatinib and nilotinib. This knowledge may contribute to identify novel targets for the treatment of metabolic bone diseases. In this topic it is we who performed whole transcriptome analysis on osteoblasts for the first time in order to understand more about the background of these molecular mechanisms. Moreover in Hungary it is we who made comprehensive literature review for the first time about the effect of tyrosine kinase inhibitors on bone metabolism.

6. Publications

Publications related to the thesis:

1. **Kirschner Gy**, Balla B, Horváth P, Kövesdi A, Lakatos G, Takács I, Nagy Zs, Tobiás B, Árvai K, Kósa JP, Lakatos P. (2016) Effects of imatinib and nilotinib on the whole transcriptome of cultured murine osteoblasts. Mol Med Rep, 14: 2025-2037. IF: 1,692
2. **Kirschner Gy**, Balla B, Kósa JP, Horváth P, Kövesdi A, Lakatos G, Takács I, Nagy Zs, Tóbiás B, Árvai K, Lakatos P. (2016) Az onkohematológiai betegségek kezelésében használt tirozin-kináz gátló imatinib és nilotinib csonthatásainak irodalmi áttekintése és a saját kutatási eredmények bemutatása. Orv Hetil 157 (36): 1429-1437.
   IF: 0,349
3. Horváth P, Balla B, Kósa JP, Tobiás B, Szili B, **Kirschner Gy**, Győri G, Kató K, Lakatos P, Takács I. (2016) Strong effect of SNP rs4988300 of the LRP5 gene on bone phenotype of Caucasian postmenopausal

women. J Bone Miner Metab, 34: 79-85. IF: 2,423

Other publications:

1. Árvai K, Horváth P, Balla B, Tobiás B, Kató K, **Kirschner Gy**, Klujber V, Lakatos P, Kósa JP. (2016) Next-generation sequencing of common osteogenesis imperfecta-related genes in clinical practice. Sci Rep, 6: 28417, 2016. IF: 4,259
2. Lakatos P, Tóbiás B, Kósa JP, Halászlaki Cs, Balla B, Árvai K, **Kirschner Gy**, Putz Zs, Dank M, Takács I. (2016) Differenciál pajzsmirigyrákok molekuláris diagnosztikája: Hol tartunk 2016-ban?. MBA, 69:(2) pp. 98-103.
3. Szili B, Bakos B, Kató K, **Kirschner Gy**, Tóbiás B, Balla B, Horváth P. (2014) A D3-vitamin-kezelés különböző adagolási sémáinak összehasonlítása. LAM KID 4: 163-168, 2014.