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MYB gene expression in GIST cancer cell lines

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Opinnäytetyössä tutkittiin *MYB*-geenin ilmentymisen merkitystä GIST-syöpäsoluissa eli gastrointestinaalisen stroomatuumorin soluissa. Opinnäytetyö on osa Heikki Joensuun tutkimusryhmän GIST-kasvaimiin liittyvää laajempaa tutkimusta. Tavoitteena oli tutkia imatinib-lääkkeen sekä MYB small interfering RNA:n vaikutusta valituissa GIST 882- ja 48-solulinjoissa. Geenin ilmentymistä ja valittujen komponenttien vaikutusta tutkittiin proteiini- ja RNA-tasolla. *MYB*-geenin arvioitiin olevan voimakkaasti linkittynyt GIST-kasvaimissa oleviin muihin geeneihin kuten esimerkiksi *KIT*-geeniin. GIST-kasvaimiin liittyvien geenien merkityksen selvittämisen tavoitteena on tuoda uutta tietoa ja näkökulmaa kasvaimien alkuperästä. GIST-kasvaimien alkuperän selvitys on olennaista etenkin uusien ja tehokkaampien hoitomuotojen kehityksessä. Tutkimus pohjautuu aiempiin RNA-sekvensaatiosta saatuihin tuloksiin *MYB*-geenin ilmentymisestä GIST-syöpäsoluissa.

MYB:in ekspressoitumista, imatinibin vaikutusta ja MYB siRNA:n vaikutusta tutkittiin työssä lähtökohtaisesti proteiinitasolla. Proteiinitasolla tutkimisessa käytettiin SDS-PAGE western blotting menetelmää. RNA-tasolla samaa geeniä tutkittiin kvantitatiivisella Real-Time PCR:n TaqMan -menetelmällä. Käytettyjä GIST-solulinjoja viljeltiin ja ylläpidettiin koko työskentelyn ajan. Kasvatusmediaan lisättiin imatinib-lääkettä tai vastaavasti transfektoitiin solut MYB siRNA:lla. Tutkimus suoritettiin imatinibilla 24 ja 48 tunnin aikapisteissä sekä siRNA transfektiolla 48 tunnin aikapisteessä. Soluista tutkittiin rinnakkaisia näytteitä joko duplikaatteina tai triplikaatteina. Saatuja tuloksia verrattiin viljeltyihin hoitamattomiin kontrollisoluihin tai placebolla transfektoituihin soluihin.

Saadut tulokset osoittivat, että GIST-soluissa ilmentyy *MYB*-geeniä. Tulokset osoittivat myös, että GIST882-linjassa imatinib ja MYB siRNA alensivat *MYB*-geenin signaalia proteiinitasolla sekä RNA-tasolla havaittavasti. Signaalin vaimeneminen nähtiin western blot-kuvissa selkeänä muodostuneen linjan häivenemisellä ja qPCR-tuloksissa numeraalisena pudotuksena. Pienen otannan vuoksi tutkimuksen tulokset ovat luonteeltaan suuntaa antavia, mutta antavat kuitenkin aihetta jatkotutkimuksiin *MYB*-geenin merkityksestä GIST-soluissa.

Avainsanat	*MYB*-geeni, GIST, imatinib, MYB siRNA, western blotting

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This thesis concentrates on the meaning of *MYB* gene expression in GIST aka gastrointestinal stromal tumour cancer cells. The thesis is a part of larger research of GIST tumours in the research group of Heikki Joensuu. The goal was to study the effects of the drug imatinib and the effect of *MYB* specific small interfering RNA in selected GIST 882 and 48 cell lines. Gene expression and the effect of selected components were studied in both protein and RNA level. *MYB* gene is estimated to be strongly linked to other genes in GIST for example to *KIT* gene.

The aim off revealing the meaning of genes linked to GISTs was to bring new information and point of view of the tumour origins to light. The knowledge of the origins of GISTs is important especially in the development of new and more effective treatments. This study is based on earlier RNA sequencing results of *MYB* gene expression in GIST cancer cells.

Expression of *MYB*, the effect of imatinib and the effect of *MYB* specific siRNA were studied primarily on protein level. For studying the protein level expression, SDS-page western blotting was used. On the RNA level same gene was studied using quantitative TaqMan method of Real-Time PCR. Used GIST cell lines were cultured and maintained throughout the study. Imatinib drug was added to the cells or the cells were transfected with MYB siRNA. The study was conducted with imatinib in 24 hour and 48 hour time points and with siRNAs in 48 hour time point. Parallel samples were studied from the cells in either duplicates or triplicates. Gained results were compared to cultured non-treated or cultured placebo transfected cells.

Results indicate that GIST cells express *MYB* gene. Moreover, results indicate that in GIST cell line 882 imatinib and MYB siRNA diminish the signal of *MYB* on protein and on RNA level noticeably. Fading of the signal was seen on western blot pictures as a clear diminish of formed line and on qPCR results as a numeral drop. Because of the small amount of samples, the results from this study are more suggestive than final. Granted, results do give subject for further studies in the field of meaning of *MYB* gene in GIST cells.

Keywords	*MYB* gene, GIST, imatinib, MYB siRNA, western blotting

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1 Introduction

The purpose of cancer research is mainly to improve knowledge about cancer. This way it is able to find out ways in preventing the occurrence of different cancers. Additionally, scientific research can improve and help to find new ways to diagnose and treat cancer more effectively. When it is able to discover improved ways to fight back cancer, there is a higher possibility to help people understand cancer and its origins better. Through open and linked global connections, we can as a society accomplish great actions towards the fight against cancer. (Cancer Research UK 2009 – 2014.)

This thesis is strongly linked to the research of gastrointestinal stromal tumours ergo GISTs and its origins. In this particular research study, the meaning of *MYB* gene expression in GIST cancer cell lines was studied. Additionally, the basic function of protein production and ways to interfere this event, were examined during the study. Preliminary results of Dr. Sihto indicated that *MYB* gene might be controlling transcription of other genes which are known to be associated with GIST tumorigenesis (Sihto 2016). The most known gene that causes GIST is so called *KIT* gene and about 85 – 95% of this kind of tumours arise from the mutation in the *KIT* gene (Sihto 2016).

The main purpose was to find ways to help preventing the formation of GIST cancer cells and understand more about how this particular tumour is shaped in the intestines. Furthermore, this study might work as a foundation in helping to find out more about genetics behind tumour formation, especially concerning the role of *MYB* gene in GISTs. The aim of this study was to answer if the GIST cells express *MYB* gene and if imatinib or *MYB* specific siRNA reduce the protein or RNA signal of *MYB* in GIST cells.

Research questions for this thesis were:

1. Do GIST cancer cells express *MYB* gene?
2. Does imatinib or MYB siRNA reduce the protein or RNA signal of *MYB* gene in GIST cancer cells?

By using cell culture, the amount of the used GIST cancer cells was multiplied. The *MYB* gene expression was detected by using western blotting. With western blotting it was possible to reveal if cells produce protein that is coded by the *MYB* gene. This protein production was reduced by blocking transcription of the messenger RNA of *MYB* with

imatinib or MYB siRNA. When the amount of studied mRNA decreases, one can assume that the amount of protein decreases in the same proportion. When the production of abnormal *MYB* controlled protein level decreases, the effects of this decreasing in cell apoptosis or in cell proliferation in GIST cancer cells could be seen.

This study was conducted to the research group of Heikki Joensuu, University of Helsinki. For the research team, the purpose of this study was to gain more knowledge of *MYB* gene function. Research group of Heikki Joensuu had already supporting evidence from RNA sequencing results concerning this thesis and these results were the foundation behind the thesis (Sihto 2016). Protein production of *MYB* in GIST cells was provisionally discovered by western blotting. Moreover, RNA sequencing results had shown that the amount of MYB mRNA decreases when for example imatinib is added to the cell culture. To verify these results western blotting was repeated with imatinib treated cells and with MYB siRNA transfected cells. The results gained from the study will hopefully improve research team's purpose of resolving the origins of GIST.

The thesis consist of background about GIST and *MYB*, introduction to used laboratory materials and methods, analysing and reporting gained results, discussion with conclusion and possible follow-up actions towards *MYB* research in GISTs. Additionally, there are six appendices related to this thesis. Information of this thesis can be used to study more about *MYB* gene expression in the further research of GIST.

2 Background

The most recognised ability of cancer cells is their way of sustaining unlimited proliferation. Normally tissues control their cell production and are able to release growth-promoting signals that can guide the cell's growth and division cycle. This release and control can help in maintaining homeostasis, keeping normal structure and controlling function of the cell. Cancer cells disturb these signals and this way 'determine' their own destinies. This chronic proliferation can be sustained in multiple ways. Cells can produce growth factor signals, they can send signals to stimulate normal tissues, and they can increase the levels of receptor proteins in the cancer cell surface and become hyper responsive towards growth factors. (Hanahan – Weinberg 2011.)

Normal cells use negative-feedback to control their homeostatic balance. Damages in negative-feedback systems are able to enhance proliferative signalling for example in

cancer cells. Increasing expression of oncogenes and their protein production signals, on the other hand, can effect directly on elevated cancer cell proliferation and in that way to tumour growth. Additionally, cancer cells have to come up with programs that negatively monitor cell proliferation. These actions are partially dependant on the cells disappeared action of tumour suppressor genes. Above all cancer cells need nutrients and oxygen and a way to lose metabolic wastes that angiogenesis provides in the form of tumour-associated neovasculature. (Hanahan – Weinberg 2011.)

2.1 GIST

GISTs are gastrointestinal mesenchymal tumours that generally embody CD117 (*KIT*) proto-oncogene protein. GIST tumours are just from 1 to 3 % of all of the GI tumours, but it is the most common soft tissue sarcoma of the gastro intestines. It is believed that GIST tumours originate from precursor cells of the interstitial cells of Cajal, which are in control of spontaneous abdominal peristaltic movements. Usually, the treatment of GIST, includes the removal of the tumour in 50% to 60% of cases. Additionally, imatinib can be used in metastatic GISTs or in GISTs that have a high risk of progression. (Lääkärin käsikirja 2014; Sihto 2016.)

2.1.1 GIST diagnosis

Generally GISTs are caused by activating mutations in the *KIT* or *PDGFRA* (Platelet Derived Growth Factor Receptor Alpha) genes. These tumours are normally found in stomach (60 %), small intestine, jejunum and ileum (30 %), duodenum (5 %), rectum (2–3 %) and from colon (1–2 %). GISTs consist of a group of neoplasms with various morphology, behaviour and genetic features. On histopathologic view, GISTs are epithelioid-, spindle-, or mixed-cell tumours. These tumours can be categorised as malignant, borderline or benign depending on its size, invasion to nearby tissues and mitotic index. (Poveda et al. 2013.)

The diagnosis of these tumours mostly relies on its histopathological features and on the immunohistochemical phenotype. Anoctamin-1 (ANO1/DOG1) is known marker for GISTs and is used in differential diagnosis of GIST. Moreover, determination of *KIT* and *PDGFRA* mutations, are connected to GIST diagnostics. These mutations are helpful to

confirm diagnose in uncertain cases. The mutations can also help determine the response of imatinib in tumours. (Gong – Li – Zhao – Zhao – Zhang 2009; Sihto 2016.)

Mostly GISTs occur in humans at the age of 60. Additionally, GISTs usually encode mutated tyrosine kinase receptors that are the most common therapeutic targets of tyrosine kinase inhibitors such as imatinib and sunitib. Wild-type versions of GISTs occur 85% – 90% on children and only 10% – 15% on adults. These wild-type GISTs are characterized with the lack of *KIT* or *PDGFRA* mutation. For these GISTs, imatinib and sunitib have been noticed to be less effective choice of treatment. Both *KIT* and *PDGFRA* encode similar tyrosine kinase receptors structurally and in GISTs mutations at those genes result in abnormal activity of proteins. These proteins have constitutive increased oncogenic signalling when they have mutated absence of their important ligands. Altered and uncontrolled kinase activity causes alternations to protein translation, cell cycle, apoptosis and cell metabolism. Metastases of GISTs are rare and are mostly seen only in the late-stage disease or in pediatric GISTs as lymph node metastases. (Foo – Liegl-Atzwanger – Lazart 2012.)

2.1.2 GIST mutations in research

Ostrowski et al. studied molecular characteristics of tyrosine kinase receptors in family three that are connected with known GIST mutations in *KIT* and *PDGFRA*. Both mutations are thought to promote early oncogenic events in similar pathways. Purified RNA from the GISTs and sequencing of mRNA was used to clarify these *KIT* and *PDGFRA* mutations. Most of the tumours possessed either or both of these mutations. Gene expression altered between two groups of GISTs at some levels for example considering angiogenesis. Their study found new molecular elements that might be involved in receptor-dependent GIST evolution and also backed up previously known information about GIST receptors. These molecular elements might be good therapeutic targets and markers of *KIT* mutation status. (Ostrowski et al. 2009.)

In Heinrich et al. research study in year 2006 these oncogenic mutation spots *KIT* and *PDGFRA* were noticed to develop new imatinib resistant secondary mutations. In the study of 147 patients with advanced GISTs, effects of imatinib was studied in a clinical environment. Samples of GISTs were from pretreatment or from imatinib-resistant tumours. Samples were biochemically analysed to identify molecular imatinib resistance

and to identify imatinib resistant secondary kinase mutations of *KIT* or *PDGFRA*. Samples were also profiled for imatinib sensitivity. Results showed that imatinib-resistant tumours had similar or greater levels of *KIT* with untreated GISTs. Secondary kinase mutations seemed to be rare in primary resistance GISTs but often found in secondary resistance GISTs. In Heinrich et al. study secondary kinase mutations were linked to reduced imatinib sensitivity compared with more characteristic *KIT* exon 11 mutations. Imatinib-resistant GISTs seem to remain dependent on *KIT* kinase activity in activation of downstream signalling pathways. Heinrich et al. proved that biochemical molecular mechanisms are in charge of primary and secondary imatinib resistance in GISTs. (Heinrich et al. 2006.)

Furthermore, primary and secondary kinase genotypes were studied in another Heinrich et al. study in the year 2008. Impact of genotypes were studied with the activity of drug sunitib due to imatinib-resistance. The study was conducted with 97 patients with metastatic, imatinib-resistant or imatinib-intolerant GISTs. Mutational *KIT* or *PDGFRA* status was determined of 78 patients with the use of tumour specimens that were obtained before and after prior imatinib therapy. Sunitib and imatinib sensitivity were biochemically profiled from kinase mutants. Clinical usefulness with the use of sunitib was seen in the three most common primary genotypes of GIST: *KIT* exon 9 in 58%, *KIT* exon 11 in 34% and a wild-type *KIT* or *PDGFRA* in 56%. The progression-free survival and overall survival rate were longer for patients with primary mutation in *KIT* exon 9 or with wild-type mutations than patients with mutation in *KIT* exon 11. Additionally, secondary mutations in *KIT* exon 13 or 14 had longer survival rates than secondary mutations in *KIT* exon 17 or 18. Sunitib's clinical effect after failure in imatinib treatment is influenced by both primary and secondary mutations which correlates to the need of optimization of the treatment for GIST patients. (Heinrich et al. 2008.)

Genetically downstream targets for *KIT* were studied in pediatric GISTs by Agaram et al. in the year 2008. Pediatric GISTs are quite rare and occur primarily in females. Typically these GISTs lack the usual mutations in *KIT* and *PDGFRA* genes, but typically the KIT oncoprotein is often overexpressed in pediatric GISTs. This reason, *KIT* downstream targets and alternations in *KIT* or *PDGFRA* gene copy numbers were investigated. Overall, 17 pediatric GISTs were studied for *KIT* or *PDGFRA* genotype and biochemical activation in *KIT* downstream targets. Pediatric GISTs proved to have a distinct transcriptional signature with overexpression of *KIT* downstream targets *PLAG1*, *FGF4*, *NELL1*,

IGF1R and *BAALC*. These signatures suggest that pediatric GISTS have different biological features than what wild-type adult GISTs have. (Agaram et al. 2008.)

2.2 Proto-oncogene *MYB*

MYB is a proto-oncogene that is located on chromosome 6q in human genome. Its main function is to encode a 72 kDa nuclear binding transcription factor. Transcription factor in question is identified to be a sequence-specific DNA-binding transcriptional regulator protein in human cells. There are three main functional areas; amino terminus as a DNA binding domain, mid portion as a transcriptional trans-activating domain and also carboxyl terminus as a negative regulator in this transcription factor. *MYB* is especially found in hematopoietic cells and *MYB* expression is particularly high in immature hematopoietic cells. Recently *MYB* has been found also from non-hematopoietic cells and from cancer cells involving lung, breast, colon carcinoma and melanoma. (Kim et al. 2008.)

2.2.1 Function of *MYB* gene

The family of *MYB* genes consist of three different types of *MYB*, which are named A, B and c-Myb. All of these genes encode proteins that are involved in transcription. This study focuses on the gene c-Myb and that particular gene is called in this thesis the *MYB* gene. The transactivation of different *MYB*s varies highly on different cell types and in promoter contexts. This variability between different *MYB*s suggests that there are also dependence with *MYB*s and other cell type specific co-factors for example interaction with other transcription factors. Gene expression of these genes is noticed to be cell cycle-regulated. If this expression is inhibited with anti-sense oligonucleotides, it may effect on cell-cycle progression, cell differentiation and cell division. (Oh – Reddy 1999.)

V-myb is a transforming homolog oncogene of *MYB* that is connected to avian myeloblastosis virus (AMV) and to avian leukaemia virus E26. These viruses are strongly connected to leukaemia. The so called v-myb genes are able to transform haematopoietic cells in tissue cultures and causes leukaemia in animals. Because the MYB protein is known to be a DNA-binding transcription factor, its oncogenic activity might be linked to its capacity to monitor specific target genes that impact cell reproduction or tumorigenesis. (Kim et al. 2008; Quintana et al. 2011.)

2.2.2 Role of *MYB* in cancer

Bell et al. studied recurring of *MYB* gene translocation of t(6;9)(q22–23;p23–24) in adenoid cystic carcinoma (AdCC) of salivary glands. High expression of transcriptional factor MYB was found in many AdCCs. Bell et al. investigated the biological and predictive significance of the increased levels of *MYB* and its known downstream targets (c-kit, cox-2 and bcl-2). 156 AdCCs samples protein expression of these genes were analysed by immunohistochemical methods. Results showed that 55% of the studied AdCC samples had increased *MYB* expression. Moreover, the combination of *MYB*+/c-kit+/cox-2+ had better survival rate than combination of *MYB*-/c-kit+/cox-2+ AdCCs. Results from Bell et al. study indicate that *MYB* might serve as a target for the management of AdCC. (Bell et al. 2011.)

In research study of West et al. *MYB* gene was additionally examined in adenoid cystic carcinoma. They also discovered that translocation t(6;9)(q22-23;p23-24) was connected to multiple cases of AdCC. Moreover, the fusion between transcription encoding *MYB* gene and NFIB transcription factor in 6 out of 11 occasions of AdCC was discovered. At that time only a potential pathogenic translocation suggested it to be a useful diagnostic advantage for differentiation of AdCC. In the study of West et al. *MYB* translocation was searched by increased levels of *MYB* expression. *MYB* translocation was discovered from one-half of the AdCC cases (49 %), but from the other salivary gland tumours there were no *MYB* translocation to be found. All in all, 65 % AdCCs have an unusual *MYB* FISH pattern, which indicates *MYB* translocation. (West et al. 2011.)

Quintana et al. studied the role of *MYB* gene in breast cancer and *MYB* connection to estrogen levels and estrogen response in breast cancer cells. Their idea was that expression of *MYB* gene is associated with the estrogen receptors (ERs) in breast tumours. The *MYB* gene reacts to the activation of ERs in certain known breast cancer cell lines such as MCF-7. MYB transcription factor was thought to be connected in the regulation of many different gene expressions that are important in breast cancer evolution and progression. Thus, when *MYB* gene is activated and starts to produce transcription factor, other genes react to this transcription factor and start for example producing breast cells in an uncontrolled way. Linkage to ERs in breast cancer may also indicate that MYB transcription factor is regulated mainly by different protein to protein interactions. These Interactions may help to guide MYB transcription factor to different targets in multiple tissues. (Quintana et al. 2011.)

Furthermore, *MYB* expression was studied in connection to vav1 signal transducer protein positive lung cancers in Ilan – Katzav research study in the year 2012. They demonstrated how the vav1 promoter affects its transcription in different histological origins because of mutations in its transcription factor binding sites. Exhaustion of *MYB* with siRNA was seen as a reduction in vav1 expression in lung cancer cells. Additionally, co-transfection of *MYB* activated transcription of vav1 promoter decreased vav1 expression. These results indicated that *MYB* is involved in lung cancer through vav1 expression. (Ilan – Katzav 2012.)

In hematopoietic cancers – such as T-cell acute lymphoblastic leukaemia aka T-ALL – *MYB* expression can work with so called super-enhancers that effect to known oncogenes for example in T-ALL TAL1 oncogene. This was discovered in research study of Mansour et al. in the year 2014. MYB transcription factor was investigated to effect an upstream super-enhancer of TAL1 oncogene. MYB binds itself upstream of TAL1 and recruits its binding partner CBP among other components of leukaemogenic transcriptional complexes. In Mansour et al. study, they reduced the activity of the mutant reporters after MYB knockdown, which indicated that the enhancer activity passed by the mutations was indeed meditated by MYB. In T-ALL samples and cells mutations that introduce MYB binding sites were at a hotspot 7.5 kb upstream from TAL1. This binding of MYB creates a super-enhancer that drives monoallelic overexpression on TAL1 oncogene. In this study Mansour et al. used new MYB antibodies in generating maps of genome-wind binding of MYB in Jurkat cells. TAL1 enhancer's mutation site analysis in Jurkat cells pointed out the precise alignment of MYB binding and binding of all the members of TAL1 complex. siRNA knockdown of MYB resulted exhaustion of TAL1 expression in both used cell lines (Jurkat and MOLT-3 cells). (Mansour et al. 2014.)

Generally, there is only little known about *MYB* in tumorigenesis in human cells. More studies and advanced techniques may be required to resolve the mysteries of *MYB*. More results may help to understand which kind of signals and regulators are important in the role of controlling the activity of MYB transcription factor. However, it is not yet absolutely clear if the MYB transcription factor regulate genes that are involved in tumour growth. (Quintana et al. 2011.)

2.3 Imatinib and siRNA

Imatinib is a drug that is used in drug therapy of GISTs, chronic myeloid leukaemia (CML) – that is Philadelphia-chromosome positive – and acute lymphoblastic leukaemia et cetera. Imatinib is a tyrosine kinase inhibitor that works by blocking the function of tyrosine kinases with abnormal enzymatic activity. Target proteins for imatinib are, among other oncoproteins, KIT, PDGFRA and BCR-ABL (connected with CML). These targets can be in charge of giving cancer cells signals to multiply without control. Imatinib helps preventing the uncontrolled growth in cancer cells. (Imatinib (Glivek) 2015; Sihto 2016; Lee – Wang 2009.)

Imatinib is characterized as an effective drug in cancer treatments and also in small-molecular targeted therapies. Nevertheless, it possess some mild adverse effects such as muscle cramps, edema, diarrhea and bone-marrow toxicity. These effects might sometimes cause patients to discontinue imatinib treatment. Despite of adverse effects, imatinib versatility on multiple diseases makes it a fine treatment option for cancers. (Lee – Wang 2009.)

siRNA is a small interfering RNA that interferes expression of protein coding genes. siRNA is a synthetic duplex RNA designed to attach identical corresponding mRNA in sequence to undermine its function. When attached, siRNAs can block the protein production. siRNAs can also knockdown gene expression by causing promoter genes methylation and chromatin condensation. Their ability to induce gene knockdown is limited to cells that are receptive to transfection of synthetic oligonucleotides. (Ross –Carlson – Brock 2007; Dharmacon 2016.)

3 Materials and methods

GIST cell lines for this study were kindly provided by Jonathan Fletcher MD from Boston, Massachusetts, United States of America. Cells were separated from human GISTs and they were molecularly characterized as GIST cells. GIST882 cell line was established from a patient with untreated metastatic GIST. This cell line expresses' a homozygous missense mutation in *KIT* exon 13. This mutation was resulted by a single amino acid substitution (K642E), in the proximal area of the split tyrosine kinase domain. In addition, GIST882 contains monosomy of chromosomes 14 and 22. Other cell line used, GIST48, was established from progressed GIST after clinical response to the given treatment

(imatinib therapy). Cell line 48 contains primary in *KIT* exon 11 missense mutation (V560D) and secondary missense mutation (D820A) in *KIT* exon 17 (kinase activation loop). (Bauer – Duensing – Demetri – Fletcher 2007; Tuveson et al. 2001; Bauer – Yu – Demetri – Fletcher 2006.)

Cell lines were cultured to the optimal stage of growth and after that protein levels of cells were studied by using SDS-PAGE western blotting. In addition, to gain more knowledge, RNA level of *MYB* was studied by performing RNA isolation and qPCR on GIST cell lines.

3.1 Cell culture

In cell culture, cells are grown in an optimal artificial environment. Cells are removed from an animal or humans and they can be removed from tissues directly. Before culturing, cells can be manipulated by enzymatic or mechanical means or they can be separated from a cell line that has already been established. The condition to grow cells vary depending on the cell type in use. The artificial environment to grow cells though contains primarily the following aspects:

- A substrate that includes essential nutrients (for example amino acids and carbohydrates)
- Growth factors
- Gases (oxygen and carbon dioxide)
- Hormones
- Regulated environment (pH, osmotic pressure, temperature)

(Thermo Fisher Scientific 2016.)

To culture cells in laboratory environment, the laboratory must have certain aspects. Laboratory is recommended to be dust free and have no through traffic that can have a harmful effect to the cells. Laminar flow hoods allow these aspects to be fulfilled. Laminar flow hoods provide also an aseptic area for working with tissues. The laboratory should also be separated from the preparation area, wash-up and sterilization places, while still being close to each other on primary cell cultures. (Freshney 2010: 25, 37.)

An artificial substrate is a base where cultured cells can grow as monolayers or as a suspension. That is why the substrate must allow cell adhesions, which allows cells to grow and spread throughout the surface. The most commonly used substrate materials are disposable plastic and glass. These materials can be pre-treated with for example nutrients that improve cell attachment and growth. (Freshney 2010: 89–90.)

Growth factors in serum stimulate cell proliferation, especially those factors that are platelet-derived (PDGF – platelet-derived growth factor). PDGF is known to stimulate growth particularly in glia cells and also in fibroblasts. Other growth factors such as fibroblast growth factors and epidermal growth factors are to be considered in cell cultures. Many of these factors are available for buying as recombinant proteins. Hormones that have a mitogenic feature, like insulin, growth hormone and hydrocortisone, are used in cell cultures. Insulin improves the up taking of glucose and amino acids. Hydrocortisone for its part, can promote cell to cell attachments. (Freshney 2010: 111.)

As for the controlled and stable environment of the cell culture, cell type specification define the used physical aspects. Commonly most cells are accustomed to grow well at pH 7.4. Carbon dioxide helps lowering the pH by dissolving into bicarbonates. Additionally, oxygen is required to maintain the cell respiration. Although, it is important to stable the oxygen level to the stage where it helps respiration but does not release toxins to the culture. The variation in osmolality is widely tolerated among different cells, but recommended osmolality in culture is between 260 mOsm/kg and 320 mOsm/kg. As for temperature, it is recommended to resemble the original source of the cells (growing human cells temperature is optimal in 37 °C). (Freshney 2010: 99–106.)

3.2 SDS-PAGE

In SDS-PAGE, proteins are examined in their denatured state. The SDS-PAGE gel itself contains SDS (Sodium dodecyl sulfate) and the PAGE gel (Polyacrylamide gel) can be used with SDS in the running buffer (PubChem 2005; BioRad 2016). Denatured state enables the estimation of protein individual molecular weight and can be an additional property to separate proteins from each other. The SDS itself is an anionic detergent that has an ability to unfold protein structures and provides them extra negative charge by binding into the proteins in high temperature. The number of negative charges and the amount of attached SDS molecules is related to the length of the studied polypeptide chain. (Hegyi et al. 2013.)

The charge-to-mass-ratio and the shape of the protein will become approximately identical to different proteins upon SDS-treatment. Hence, the used SDS gel is able to separate individual polypeptide chains merely by their size. This way SDS-PAGE basically separates proteins by their molecular mass (size is a linear function to mass). Proteins that have a known molecular mass can be used to form a calibration curve which helps to estimate the unknown molecular masses of other proteins. (Hegyi et al. 2013.)

3.3 Western blotting

Western blotting is a technique that is used to separate wanted proteins based on a mass of proteins. The technique is based on transferring proteins from the separating gel onto a supporting matrix. This blotting style allows for fast staining and for destaining protocols of the separation of proteins. In western blotting, protein samples are separated in a gel through gel electrophoresis and after that electroblotted onto a support matrix. Once this is done, proteins can be labelled with anything that is able to selectively bind into peptides to identify the presence or absence of specific spots. This detection of proteins can be reached via an enzyme fit for use with a changing ways of colorimetric, chemiluminescent or fluorescent substrates. (Walker – Rapley 2005: 43.)

After the gel electrophoresis, protein transfer from the gel is accomplished by using an electric potential through the gel and the membrane. The applied membrane can be nitrocellulose, nylon or polyvinylidene difluoride (PVDF) base. This membrane provides much more stable environment than the gel, making later manipulations considerably easier. Electroblotting is the most common western blotting technique, but also techniques such as capillary blotting can be used. (Walker – Rapley 2005: 43–44.)

PVDF membrane tides proteins to itself via dipole and hydrophobic interactions. The PVDF membrane does not let proteins through and is less fragile than for example nitrocellulose (PVDF Membranes for Western Blotting. 2015). For electroblotting the proteins from gel to the membrane, a so called sandwich is built. For the ‘sandwich’, gel and the membrane are placed between filter papers and foam pads. Pads and paper are so light and spongy materials that electric current can easily transfer through them. They also protect the PVDF membrane and the SDS-PAGE gel. Filter papers and foam pads are damp in transfer buffer and the PVDF membrane is activated before use in methanol for one minute. (Western Blot Transfer Methods. 2015.)

Following transfer to the membrane, finding of the target proteins is commonly accomplished by using specific antibodies. The implantation of these specific antibodies to their target proteins can be performed by using methods that are based on indirect or direct immunoassays. Immunoassay methods include the using of multiple labels that are conjugated to an antibody. In direct method, the signal of these labels is noticed directly when the antibody attaches itself to the protein that is looked for. When in indirect immunoassay methods, the signal can be achieved by using enzymes that can act on particular substrates. (Walker – Rapley 2005: 46.)

3.4 RNA isolation

Generally RNA is separated from cells to transform it into cDNA. RNA is degraded quite easily especially when enzymes called RNases are present. RNases are enzymes that break RNA into pieces in cells so that the parts of RNA can be reused to build new RNAs. The used materials should be handled with reagents, such as guanide thiocynate, that inactivates RNases. Overall, RNA should be handled on ice bath to prevent any extra biochemical reactions. RNA can be isolated for example from tissues, cultured cells, plants or from bacterial cells. (Suominen – Pärssinen – Haajanen – Pelkonen 2013: 108–109.)

In silica column method of RNA isolation RNA is attached to silica membrane in high salt concentration. RNases can be inactivated by specific lysis buffer that contains for example chaotropic ions. Buffer also creates an atmosphere where RNA can bind to silica membrane. Binding of DNA is prohibited by rDNase that binds to the silica membrane instead of DNA molecules. Washing the membrane with high concentrated liquid removes unwanted particles such as salts, metabolites and other macromolecular particles, but keeps the RNA attached to the column. Pure RNA is removed from the silica membrane with low salt concentration RNase free water. (RNA isolation 2014.)

After the isolation of RNA from cells or tissues, the purity of the gained product should be measured. Purity measurement can tell the amount of isolated RNA and possibility of contaminations (for example proteins) in the eluted sample. The most common measurement technique is based on nucleic acids ability to absorb in 260 nanometres. Purity is calculated by preparing the gained results on 280 nm wavelength. Pure RNA the calculated purity is 2.0 absorption in 260 nm wavelength. (Suominen et al. 2013: 110–111.)

3.5 qPCR

PCR ergo polymerase chain reaction is used to multiply known DNA regions. The method is based on DNA polymerase enzyme that can multiply DNA sections on high temperature and on two specific primers that define the multiplied DNA section. In real-time PCR the creation of wanted nucleic acid can be monitored live while the reaction proceeds. Real-time PCR is based on fluorogenic tracer. Tracer's fluorogenic signal multiplies when attached to the finishing two strand PCR product. To get quantitatively measured amount of PCR product, this gained fluorogenic signals is used to calculate the amount of wanted product in the used sample. Real-Time quantitative PCR ergo qPCR the gained PCR product can be quantified by using relative or absolute methods. In the more common relative quantitation the amount of the target gene is compared to the amount of reference gene. (Suominen et al. 2013: 153–154, 166–170.)

For real-time PCR, TaqMan method focuses on fluorogenical features of the used probe. This probe contains a fluorescent reporter dye, which is attached to its 5' end, and a quencher dye that is linked to its 3' end. When present of the target nucleic acid sequence, TaqMan probe anneals downstream from primer site and is so called cleaved by the 5' nuclease activity of polymerase enzyme in the extension phase of the real-time PCR. While the probe is being intact in the sequence chain, fluorescence resonance energy transfer particle appears and the fluorogenic emission of the 5' reporter dye is reduced by the quenching dye. Cleavage of the used probe by polymerase enzyme Taq during the PCR divides the two dyes of each other. This action strengthens the fluorescence from the reporter dye. Moreover, cleavage detaches the probe from the target sequence, letting the primer extension to carry on to the end of the template strand. This way it does not interfere with the exponential accumulation of the wanted PCR product. In each cycle, additional reporter dye molecules can be cleaved from their probe, which leads to increased fluorescence intensity amount to the proportion of amplicon produced. This increased fluorescence emission can be detected by special modified thermocycler computer software. (Arya et al. 2005.)

Computer calculates the fluorescence emission by using relative quantification. In relative quantification mRNA levels of gene samples are determined and expressed relatively to the levels of calculated internal RNA control. This used control reference gene can be so called housekeeping gene which can be co-amplified in the same tube or can

be amplified in a separate tube. Relative quantification, therefore, does not need any standards with known concentrations and the reference can be any transcript that has a known sequence. Quantification of this kind is merely based on the expression levels of a target gene compared to a reference gene. Calculations can be based for example on the comparison of the crossing points and threshold values at a constant level of fluorescence. (Quantification strategies in real-time RT-PCR n.d.)

The used template for PCR is DNA. When multiplying RNA, it must first be transferred into DNA. In reverse transcription PCR, RNA is transferred with reverse transcriptase into complementary DNA ergo cDNA. This can be done in multiple ways but the results in all of them is two stranded cDNA that is formed of using one stranded RNA as a base. (Suominen et al. 2013: 170.)

3.6 Protocol

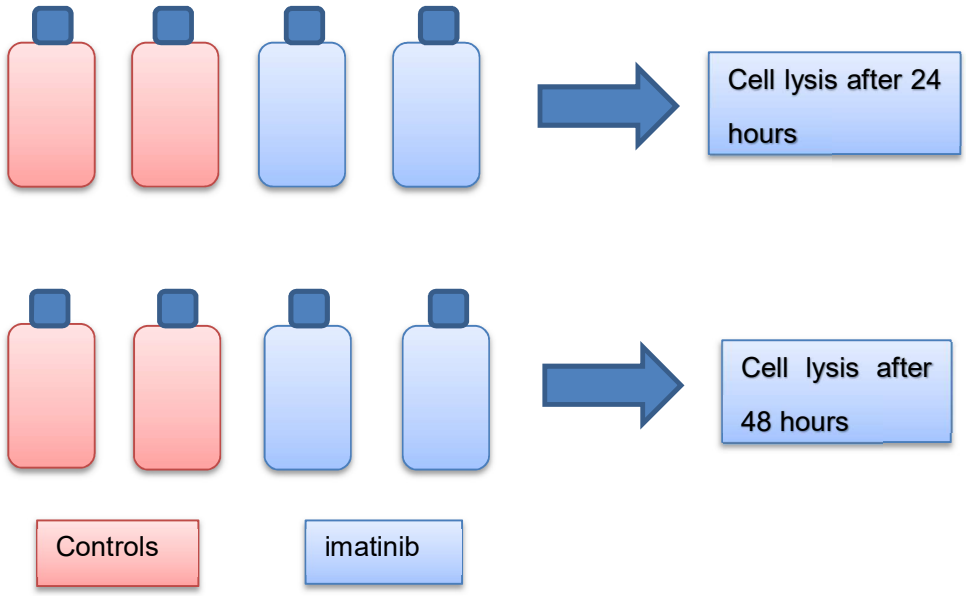
The idea behind this study was to demonstrate *MYB* expressions possible diminish on protein level by effect of imatinib or *MYB* specific siRNA. Also RNA level was studied. Used GIST cell lines, line 48 and line 882, were grown in cell culture as monolayers in plastic flasks at +37 °C and in 5% carbon dioxide. Reagent and materials used in this study are demonstrated in Appendix 1.

Line 48 was already being maintained in T75 flask by the research group. Line 882 cells were picked up from -150 °C freezer and removed from there to grow in T75 sized flask (protocol demonstrated in Appendix 2). Cells were maintained before dividing them into T25 sized flasks by changing their medium regularly and dividing them if needed (confluence being round 70% to 80%). Protocols demonstrated in Appendix 2. Confluence is the amount that cells cover the base of the bottle. As a cell growth medium, RPMI-medium base was used. To medium base inactivated fetal bovine serum, penicillin and streptomycin glutamine were added. In case of siRNA transfection, cells were divided from T75 flasks to 6-well microtiter plate or into T25 flasks.

3.6.1 Imatinib treatment

Both cell lines were divided into smaller T25 flasks at relation 1:4 (divided from 1ml cell suspension in other words 250 µl was added to each bottle from 1 ml). Cells were studied

in two different periods of time: after 24 hours and after 48 hours from the adding of the drug to the cell culture. For comparability and reliability, two flasks for each cell line contained control cells (no drug added) and two flasks contained 0,5 µM imatinib (diluted to the used RPMI medium). For 24 hour analysis, there were four flasks for each cell line, and for 48 hour analysis, there were also four bottles for each cell line. Altogether, sixteen T25 flasks were used (presented in Picture 1.).



Picture 1. Imatinib treated cell line

Before dividing cells optimal confluence should be from around 70% to 80% so that cells are in an optimal growing stage. Used cell lines were about that. Line 882 was closer to 90%. Each T25 flask contained 5 ml of medium and 250 µl of cell suspension. Cell suspension was made to the medium in use (1 ml of medium). Cells were cultured for 24h or 48h with or without the imatinib drug. Before the medium was changed (with or without the drug) confluence was in 48 about 50% and in 882 about 90% (line 48 cells were observed to grow much slower than the line 882).

Cell lysis was performed after the 24 hour mark and after the 48 hour mark for the cells. RIPA buffer with added phosphatase inhibitor and protease inhibitor was used as a lysis buffer. Detergents in RIPA buffer dissolve cell membranes and that way break the cell

structures (Overview of Cell Lysis and Protein Extraction. 2015). Added inhibitors prevent different cell enzymes (such as proteases) from working and specific detergents are used if for example only the nucleus envelope needs to be broken (Overview of Cell Lysis and Protein Extraction. 2015). The amount of lysis buffer was decided by the confluence of the cells at lysis moment (amounts of lysis buffer used in Appendix 5). Lysis was performed on ice bath with +4 °C RIPA buffer to prevent denaturation of proteins and also preventing any extra biochemical reactions in cells. Cells were detached from the T25 flasks with cell scraper and moved to 1,5 ml Eppendorf tubes. After lysis, cells were furthermore scattered with sonic soniprep 150 machine. The machine scattered the cells by using high pitch sound that broke cells and separated the proteins out to be used and measured. Between cell scattering and before making western samples cells were stored at -70 °C freezer. Working protocol for cell culture is demonstrated more thoroughly in Appendix 2.

Now ready to use samples of GIST cells, the protein level of the samples was measured. Protein measurement was conducted with Pierce™ BCA Protein Assay Kit by Thermo Fisher Scientific with colorimetric absorbance detection. For BCA aka bicinchoninic acid assay measuring, samples were diluted to RIPA buffer (882 1:10 and 48 1:2). Dilution was determined by the amount of cells there were before lysis in cell culture. Measured samples were compared to standard sample dilution series for determining the total amount of protein in the actual samples. The standard sample dilution series was provided in the assay kit and diluted according to the given instructions of the kit. For measuring, 96-well microtiter plate was used and from each Eppendorf tube duplications were made. Ten microliters of wanted sample and 200 µl of BCA reagent was used. After adding the reagent, plate was left to +37 °C to incubate for half an hour. After incubation samples were measured. Because of pipetting errors of the first time around, samples were remeasured on the next day to get more reliable results. Final and used protein measurement are presented in Appendix 5.

Using the absorbance, the amount of total protein level of the samples was able to be calculated. Using this information, it was able to determine the microliter amount in 25 µg of proteins in each sample. Using that information it was able to dilute the samples to Laemmli and β-mercaptoethanol. The bromophenol blue in Laemmli buffer colours the samples with deep purple colour and β-mercaptoethanol denatures the proteins of the

sample by reducing disulphide bonds between peptides in protein structure (Sigma-Aldrich 2016). Samples were diluted on ratio 1:1. After the preparation, samples were kept in a -70 °C freezer for 5 days.

At the beginning of western blotting for imatinib treated cells, samples were melted on ice bath. This was important so the protein in the samples would not break before gel electrophoresis. Samples were loaded onto 10-well/ 50 µl per well SDS-PAGE gel. The map for the wells and loading of the samples is presented in Picture 2. Samples run in gel electrophoresis for one hour and fifteen minutes at 100V. After the run, the gels were prepared for the blotting part of western blotting. Proteins were electroblotted from the gels to the protein membrane PVDF according to western blot protocol (Appendix 3). The samples were left in transfer buffer over the night to electroblot in 0.15 A in +4 °C cold room.

GIST48

Ladder	ctrl 1 D1	ctrl2 D1	ctrl1 D2	ctrl2 D2	IM1 D1	IM2 D1	IM1 D2	IM2 D2	Pos. ctrl

GIST882

Ladder	ctrl 1 D1	ctrl2 D1	ctrl1 D2	ctrl2 D2	IM1 D1	IM2 D1	IM1 D2	IM2 D2	Pos. ctrl

Picture 2. Sample map on SDS-PAGE gels

On the second day of western blotting, ‘sandwich’ was opened and the membranes were soaked in Ponceau S solution for one minute. Ponceau S solution colours proteins so that the blotting is visible from the gels on to the membranes and can be immunologically detected. After the colouring, membranes were washed in washing buffer (TBS+tween) for one minute and then left for one hour in 5% milk for blocking of the protein action. Fat free milk and its albumin proteins attach to empty spots in the membrane preventing unspecific antibody attachments on to the membrane later on in the western blot protocol (Sihto 2016). This way the wanted specific protein has better chances to be visualised from the membrane. After the blocking, membranes were washed at 3 times for 5 minutes per time. To the clean membranes antibody action was started to detect specific

MYB protein from the membranes. First, primary MYB antibody was added to the membranes. Primary antibody clings onto protein by making protein-antibody complexes. Primary antibody was left over night to cling onto proteins at +4 °C cold room.

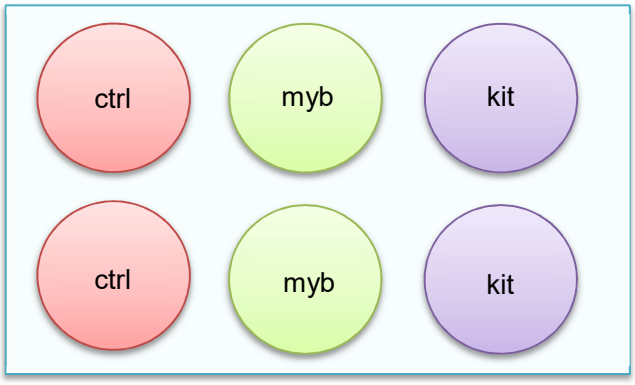
After the primary antibody, the membranes were again washed for 3 times at 5 minutes per time. The secondary antibody (rabbit anti-mouse IgG) to enhance the signal of the formed protein-antibody complexes, was added on top of the membranes and left for one hour at room temperature. The secondary antibody was labelled with HRP aka horseradish peroxidase which substrate is chemiluminescent. This way secondary antibody enhances the chemiluminescent signal of primary MYB antibody. After the secondary antibody and new washings, chemiluminescent substrate for detecting the signal of complexes was added to the membranes for 5 minutes. Chemiluminescent signal was detected after light exposure by x-ray films and the magnitude of the reaction is directly correlated to the amount of protein in the sample. The working protocol is demonstrated more thoroughly in Appendix 3.

Because the unique behaviour of the MYB protein, it had to be tested in different concentration of antibodies and in different light exposure times to detect the bands of the membranes. The result was eventually gained by using 1:3 femto chemiluminescent substrate (Thermo Fisher Scientific – Supersignal West Femto Maximum Sensitivity Substrate) and 2:3 pico chemiluminescent substrate (Thermo Fisher Scientific – Supersignal West Pico Chemiluminescent Substrate) mixture for five minutes per membrane. The exposure times varied from 30 seconds to 45 seconds. Also, 1:500 primary antibody and 1:10 000 secondary antibody were used.

3.6.2 siRNA transfection

As mentioned previously, GIST cells for transfection of the siRNAs were first divided on 6-well microtiter plate. Confluence was, before dividing the cells, in both cell lines round 90 %. Cells were counted by using Bürker chamber and diluted in Trypan blue solution that stains cells. Four A-squares were counted and then the average of the cells in one square was used in calculating the total sum of cell in one milliliter suspension. 600 000 cells was placed per well of 882 cells and 700 000 cells of 48 per well. All in all, one 6-well plate was used per cell line. Media for the cells was specially made antibiotic-free, because of the toxicity of penicillin to cells and penicillin's negative impact in transfection

effectiveness (Factors Influencing Transfection Efficiency. 2015). The 6-well plate is demonstrated in Picture 3.



Picture 3. 6-well microtiter plate for siRNA transfection

Transfection of the siRNAs for the cells was started with cell starvation. Starvation was performed by using Opti-MEM solution that lacked every substance for cells to grow and to use. Cells were left to starve for six hours and after that siRNAs were transfected. The starvation helps siRNAs to transfect, because after the starvation cells are more eager to phagocyte substances to themselves. Before the transfection, siRNAs were diluted to Opti-MEM solution and mixed with Lipofectamine that was also diluted to Opti-MEM solution. Lipofectamine creates a vehicle for the cells to transfect more easily through cell membrane (How Cationic Lipid Mediated Transfection Works. 2015). For the transfection, Opti-MEM was removed and antibiotic free growth medium was placed to the 6-well plate. Medium worked as a transfection and growing base, when siRNAs were added to each well. In this study control siRNA (called scramble that is known transfect cells without targeting genes and that way does not prevent any gene expression), MYB siRNA and KIT siRNA were used. Control and KIT siRNA were added to confirm the result from MYB siRNA. Protocol for transfection of the cells is demonstrated in Appendix 2.

After 48 hours from the transfection, cells were removed from the 6-well plate by cell lysis into Eppendorf tubes and structures of the cells were broken by sonic soniprep 150 machine as described above. The same BCA measuring and western blotting samples were made from siRNA cells as from imatinib treated cells. The western blotting protocol for siRNA did not vary much from the imatinib treated cells. For each cell line, own SDS-PAGE gel was used and the same concentration on antibodies also. Unfortunately, siRNA transfection had to be repeated with T25 cultured flasks due to lack of cell material

from the 6-well plate. Working protocol for western blotting is demonstrated in Appendix 3.

3.6.3 RNA protocol

To confirm the *MYB* expression on RNA level, RNA needed to be isolated from GIST cell lines 48 and 882. Also to confirm the effect of imatinib and siRNA in these cells, imatinib treatment and transfection of the siRNAs had to be performed. To get enough cells for the isolation, cells were divided to 24-well microtiter plate (demonstrated in Picture 4). Also, as for control, MDA-MB-361 breast cancer cell line was used. RNA was isolated by using the silica method of Machery-Nagel's RNA isolation (RNA isolation 2014). The purity of the isolated RNA and the concentration of the RNA was measured with Thermo Scientific Nanodrop 2000 Spectrophotometer.

48 Ctrl 1	**48 Ctrl 2**	**48 Ctrl 3**	**882 Ctrl 1**	**882 Ctrl 2**	**882 Ctrl 3**
48 imatinib 1	**48 imatinib 2**	**48 imatinib 3**	**882 imatinib 1**	**882 imatinib 2**	**882 imatinib 3**
48 siRNA Ctrl 1	**48 siRNA Ctrl 2**	**48 siRNA Ctrl 3**	**882 siRNA Ctrl 1**	**882 siRNA Ctrl 2**	**882 siRNA Ctrl 3**
48 siRNA MYB 1	**48 siRNA MYB 2**	**48 siRNA MYB 3**	**882 siRNA MYB 1**	**882 siRNA MYB 2**	**882 siRNA MYB 3**

Picture 4. Cell culture in 24-well microtiter plate for RNA isolation

To carry out quantitative PCR, the isolated RNA had to be transferred into complementary DNA. Reverse transcriptase enzyme can translate RNA particles to double stranded DNA. The amount of the used template was determined by the amount of the pure RNA. For example, if there were under 10ng of RNA 15µl of template was used. The translation was carried out with Dyad Disciple by Thermal Cycler. The purity and concentrations of RNA are demonstrated in Appendix 5.

Program:

- Incubation +25 for 5 min
- Incubation +46 for 20min
- Incubation +95 for 1min
- Incubation +4
- Ending

Translated cDNA was then ready for PCR. For the PCR, a reaction mix or PCR mix was made. The mix included buffer, forward primer, reverse primer, probe and sterile water (demonstrated in Appendix 4). Different mixtures were made for *MYB* and for the used control gene *G6PD*. The samples and the reaction mix were pipetted in to three different 96-well plates as triplicates of each sample. 96-well microtiter plate plan for qPCR is demonstrated in Appendix 5. For quantitative PCR, 51 PCR rounds per plate and Taq-Man method was used. The qPCR was performed by LightCycler 480 Roche Diagnostics Oy machine.

4 Results

Gained results indicate first of all that *MYB* gene is expressed in these studied GIST cell lines. Furthermore, the drug imatinib and MYB siRNA reduces MYB protein and MYB RNA levels in these cancer cells, at least in cell line 882. This reduced development on protein level is visually seen in western blot picture results and on RNA level calculated from qPCR results.

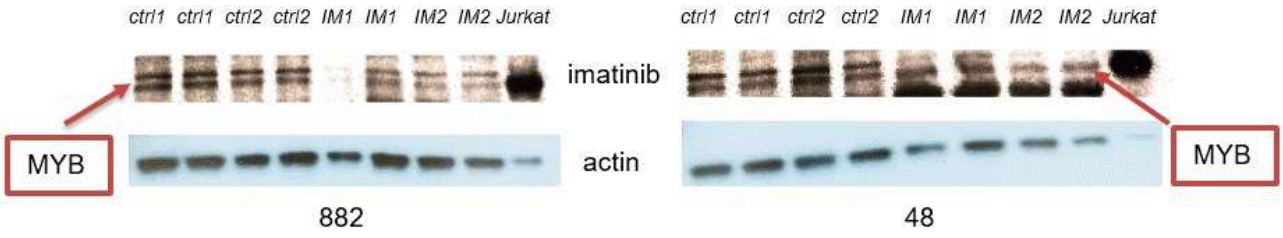
4.1 Western blot

Western blot pictures were taken after 45 seconds of exposure and after 30 seconds of lightning exposure. The clearest image was captured on 45 seconds in imatinib western blot membranes and in 30 seconds of siRNA transfected western blot membranes. It was known beforehand that *MYB* produced protein's molecular weight is seen in the pictures round 80kDa and that way it was able to detect it from unspecific bands of other proteins. The scale of different kDa was created by using ladder bands that marked different spots of kDa. Also, for control jurkat that has the same 80kDa spot to stop in western blot was used.

4.1.1 Imatinib treated cells

For imatinib western membranes, non-treated cells as controls with the imatinib treated cells were used. That way it was possible to determine the wanted specific band of MYB protein with the ladder bands and with the control band Jurkat. As shown in Picture 5, the band is the lower one of two closely settled bands. In cell line 882, the drop or fading of the band in the imatinib treated cells is clearly visible in 24 hour bands (IM1) and in 48 hour bands (IM2). Actin protein was also blotted to make sure there were enough and evenly protein loaded in the wells in the early stage of western blotting. Unfortunately, in the first of 24 hour bands of line 882, there was not enough protein loaded so the picture seems over lightened.

In cell line 48 this fading of bands is not as clearly seen as in 882. The troubles of the cell culture in 48 and getting cells co-operate in the wanted way was not as successful as was hoped. For that reason, results are based merely in the results of cell line 882 not in the cell line 48.



Picture 5. GIST882 and GIST48 imatinib treated western blot

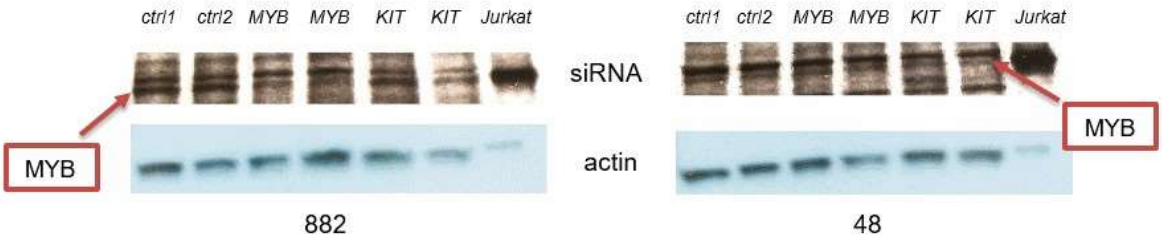
4.1.2 siRNA transfected cells

In siRNA transfection, control siRNA, MYB siRNA and KIT siRNA transfection were used. Ladder and Jurkat cell lysate were used to identify the right MYB band. Control, scramble, was there to work as negative control just to see that MYB protein band existed in cultured GIST cells. KIT siRNA was there to work as the positive control, where it was known to fade because imatinib effect is mediated through KIT receptor in GISTs.

As seen in Picture 6, the lower band is the MYB protein band and there is a slight fading in the MYB siRNA (marked as MYB in the picture) bands compared to the negative control in cell line 882. As for the KIT siRNA, the band did not fade as it was estimated to.

There might have been something wrong in the KIT siRNA that it did not work here as supposed to. Although, in the earlier footage (not shown here), the fading of KIT was clearly seen in transfected cells. Those pictures were otherwise unusable due to too many unspecific bands in the western blot picture. In Picture 6 is also seen the fact that in cell line 48 there are not any clear fading of the bands. Mostly this results from same reasons as in imatinib treated 48 GIST cells.

Unfortunately, the western blot picture from the siRNA membranes was first full of unspecific bands and was too unclear to determine the wanted MYB band could not be clearly seen. For this reason the transfection was redone with T25 flasks with the same protocol to get more cells and more protein for western blotting to gain proper results. The result here are from the T25 flasks.



Picture 6. GIST882 and GIST48 siRNA transfection western blot

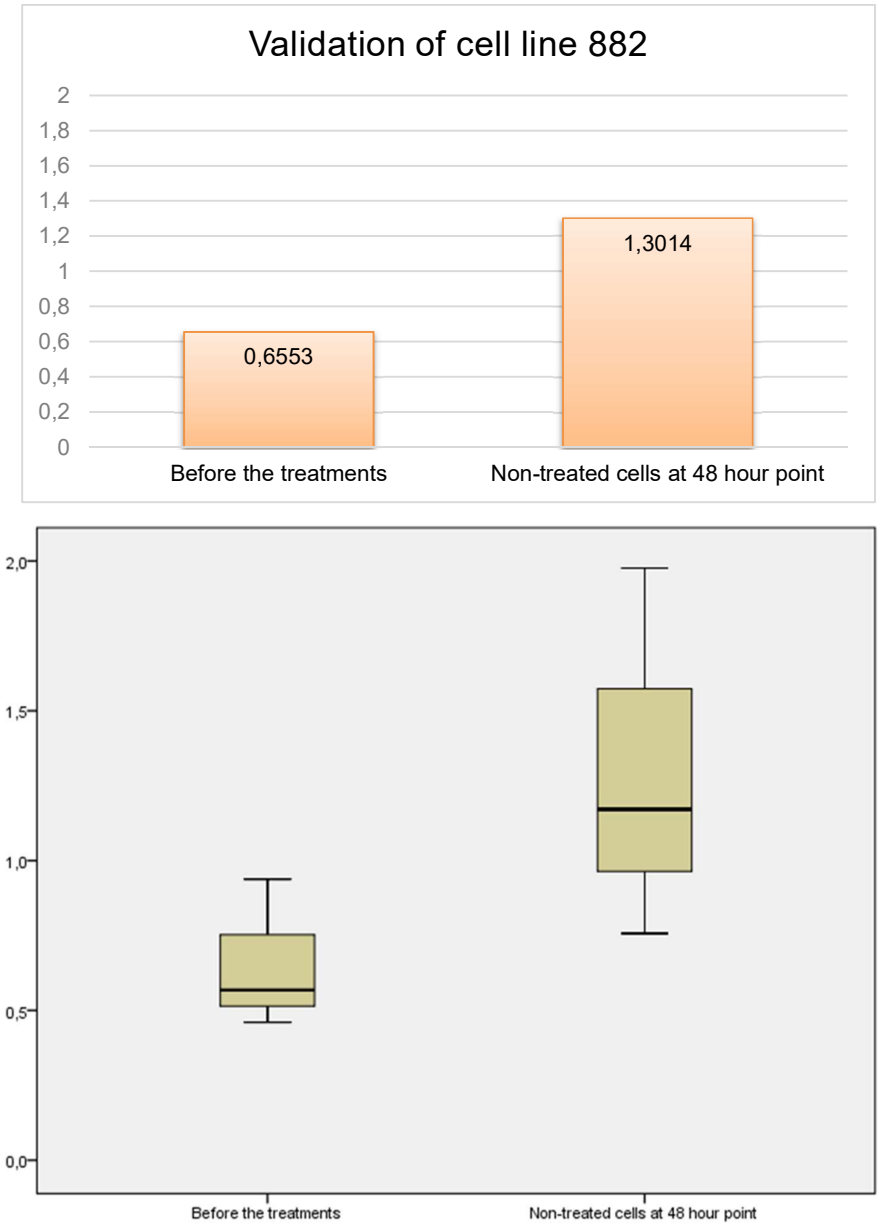
4.2 qPCR

RNA level results were gained from quantitative PCR. From each differently treated cell group, there were duplicates or triplicates used in qPCR. Because of the minor amount of studied subjects, no statistical analysis could be performed to the results. Nevertheless, the results were gathered as graphic presentations. Vertical scale in the graphs represent the mathematical figures of *MYB* gene's RNA compared to control *G6PD* gene's RNA as a relative presentation. Figures from the qPCR are demonstrated in Appendix 5.

4.2.1 GIST 882

In the shown graphics there are two groups compared with each other. For validating, the cells were separated before any treatments from the 24-well plate (left column in the

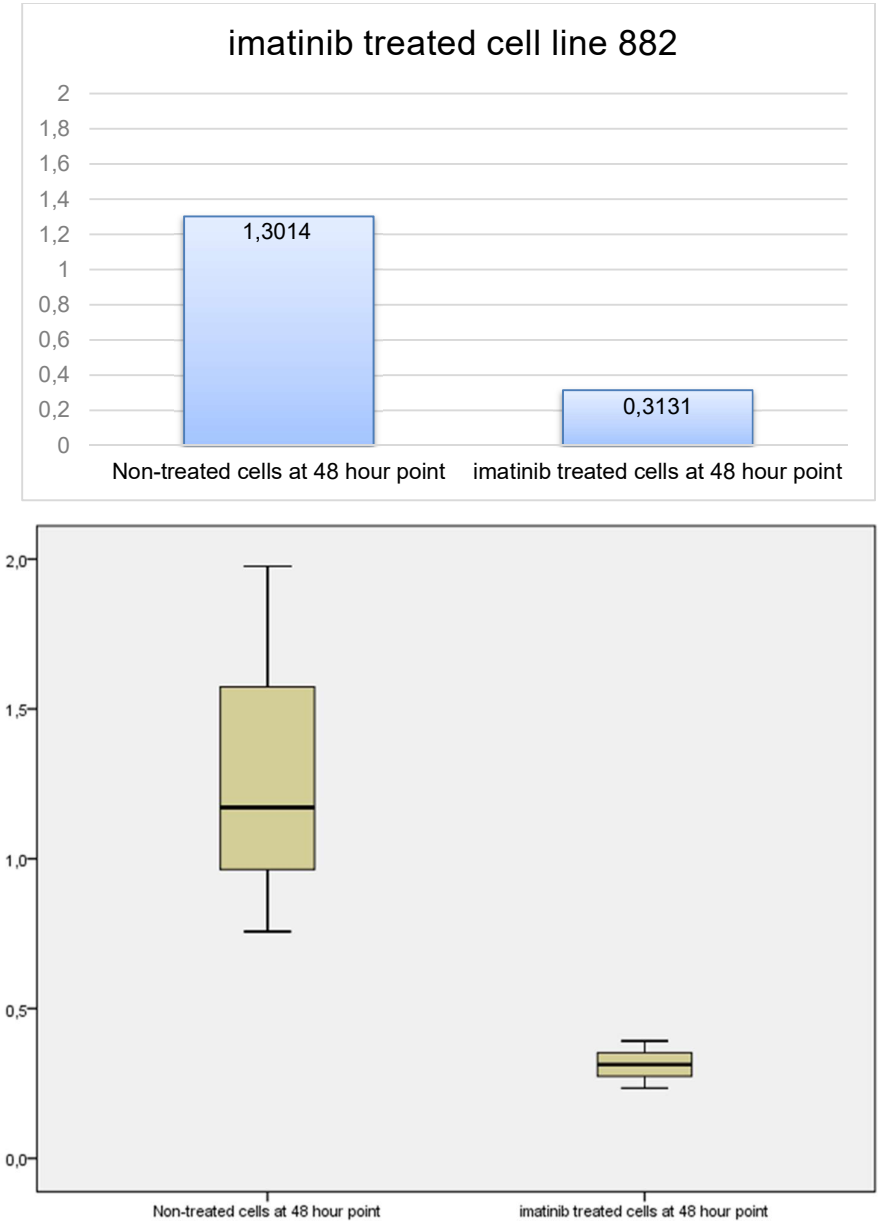
graphics) and after 48 hours of cell culturing (right column in the graphics). For the validation to be accurate, the amount of *MYB* produced RNA should have been similar between compared groups. This way would have been proved that the *MYB* produced RNA is standard on non-treated and in non-transfected cells in a cell culture. Unfortunately, because of the difficult process to get the cells lysed carefully, there were not enough cells from the before treatments point. For this reason, demonstration in the shown graphics is not acceptable for proper validation. Instead of two compared groups being on the similar level, there is more used cells and therefore more *MYB* response in 48 hour control. This way the validation did not work as it should've worked. Gained *MYB* results were proportional to the housekeeping gene *G6PD* that has a known standard expression. Validation for line 882 cells is demonstrated in Picture 7 as a histogram and as a box plot. Scale in histograms is from 0 to 2,0 and in box plots differs depending on graph.



Picture 7. Validation graphic presentation for line 882 cells. For validation, before treatments and non-treated cells on 48 hour mark, were compared with each other. Mean values, of RNA result groups, were compared with each other. Validation is demonstrated as a histogram and as a box plot on a same scale. Validation is not accurate, because the amount of MYB produced RNA is different in compared groups.

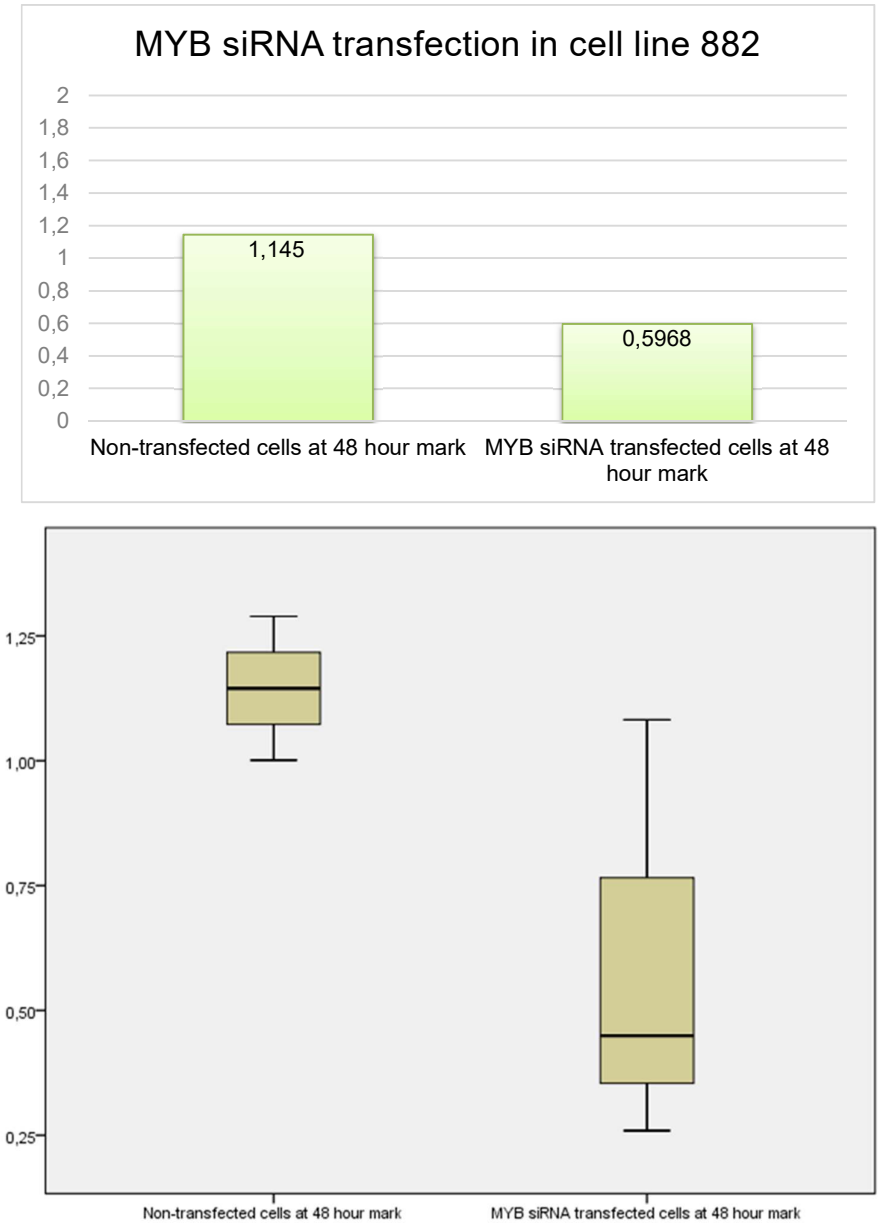
In the graphic of non-treated cells at 48 hour mark and imatinib treated cells, there is numeral decreasing of *MYB* produced RNA. Therefore, it can be said that imatinib has decreased the expression of *MYB* in RNA level in GIST cell line 882. On figures the drop of *MYB* produced RNA is from 1,3014 to 0,3131 after 48 hours of cell culturing. The effect of imatinib to GIST cell line 882 is seen in Picture 8. The left column represents non-

treated cells after 48 cell culturing and the right column represents imatinib treated cells after 48 hours of cell culturing. The same decreasing is represented as a histogram and as a box plot.



Picture 8. Graphic presentation of effect of imatinib for line 882 cells. Non-treated cells on 48 hour mark and imatinib treated cells on 48 hour mark, were compared with each other. The imatinib treatment decreased the amount of MYB produced RNA significantly from 1,3014 to 0,3131 RNA. Mean values, of RNA result groups, were compared with each other. Effect of imatinib to cell line 882 cells is demonstrated as a histogram and as a box plot on a same scale.

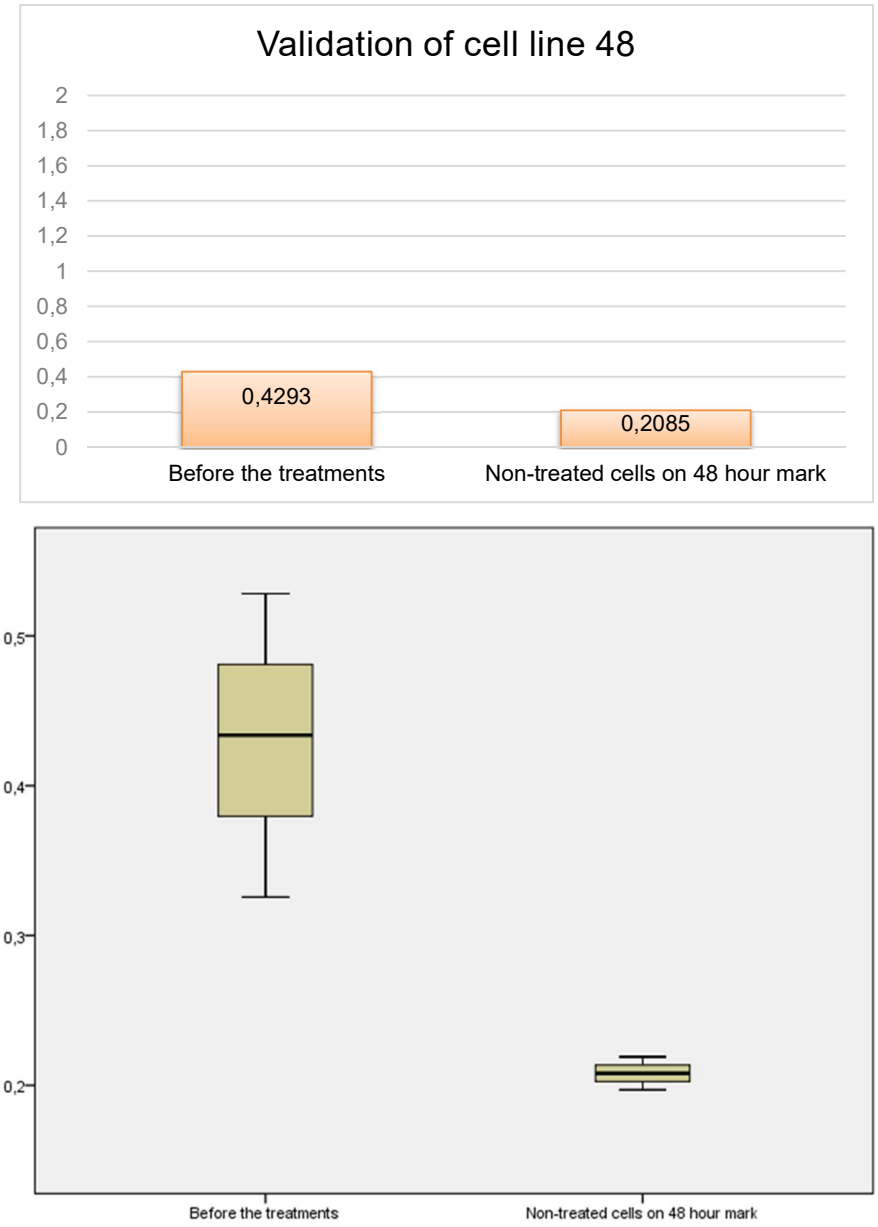
As for the effect of MYB siRNA for cell line 882, non-transfected cells on 48 hour mark and MYB siRNA transfected cells on 48 hour mark were compared with each other. The graphic shows that MYB siRNA decreases the amount of RNA compared to non-transfected line 882 cells. Non-transfected cells on 48 hour mark are represented on the left column and MYB siRNA transfected cells on 48 hour mark are represented on the right column in the graphics. On figures this decrease or drop of the amount of RNA is from 1,145 to 0,5968. The effect of MYB siRNA in cell line 882 is demonstrated in Picture 9. The same decreasing is represented as a histogram and as a box plot.



Picture 9. Graphic presentation of effect of MYB siRNA for line 882 cells. Non-transfected cells on 48 hour mark and MYB siRNA transfected cells on 48 hour mark, were compared with each other. The MYB siRNA transfection decreased the amount of MYB produced RNA significantly from 1,145 to 0,5968 RNA. Mean values, of RNA result groups, were compared with each other. Effect of MYB siRNA to cell line 882 cells is demonstrated as a histogram and as a box plot. Box plot has a different scale compared to histogram.

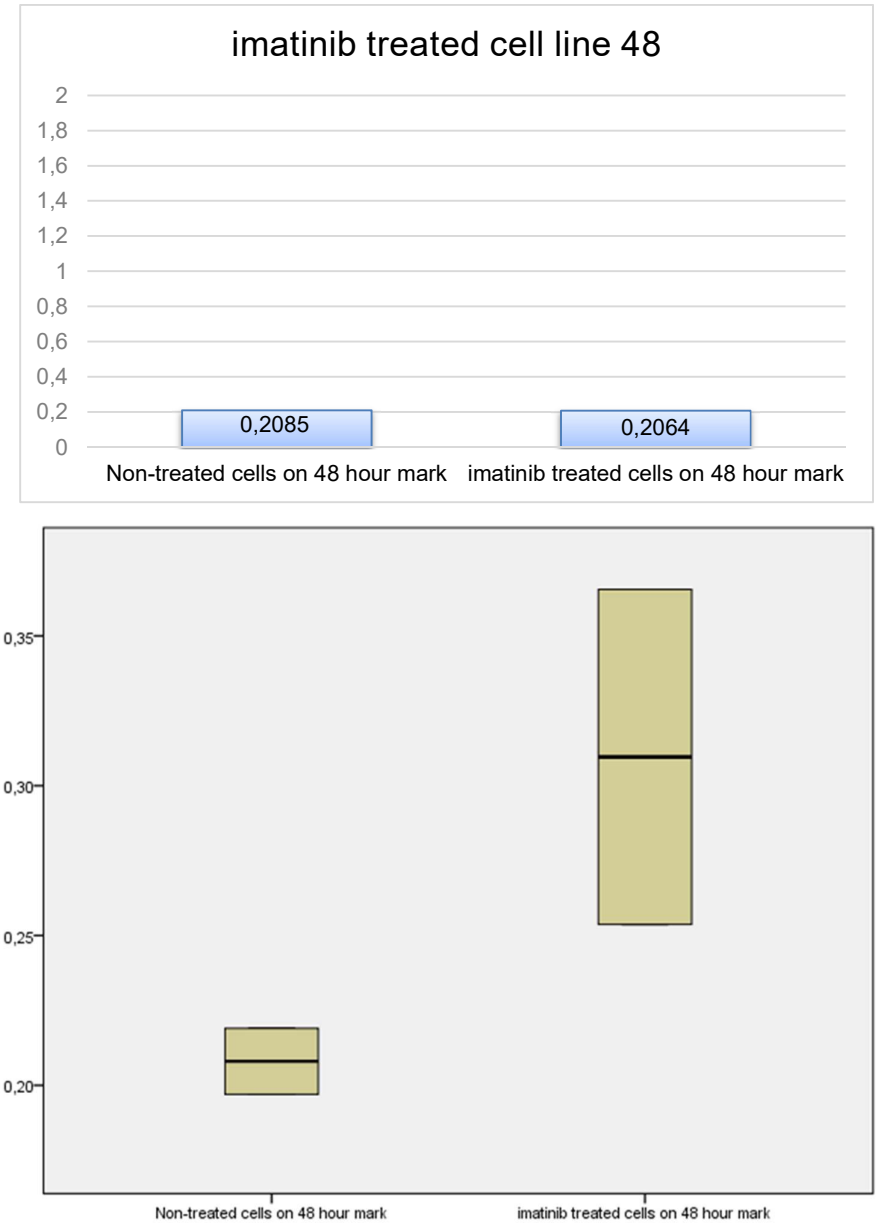
4.2.2 GIST 48

In validation for cell line 48 cells were compared in before treatments group with non-treated cells on 48 hour mark. In line 48, cells were easier to remove from the before treatments plate (than in line 882), but the amount of cells decreased from unknown reason after 48 hours of culturing. This might have affected of the amount of cells that were lysed in 48 hour cell culture 24-well plate. Overall, there were not much line 48 cells to begin with due to poor cell growing of line 48 cells in 24-well plate. This might indicate that the measured amount of *MYB* produced RNA is larger in before treatments mark than in non-treated 48 hour in mark in the validation graphic. Cell amount is not usually the reason behind qPCR results, because in relative qPCR studied gene (*MYB*) is compared to housekeeping gene (*G6PD*). Results are a reflection from the ratio between these two genes. Nevertheless, in this case the validation did not work as it was intended to work. Validation therefore should not be trusted neither in line 882 cells nor in line 48 cells. Validation for cell line 48 is demonstrated in Picture 10. Validation is represented as a histogram and as a box plot.



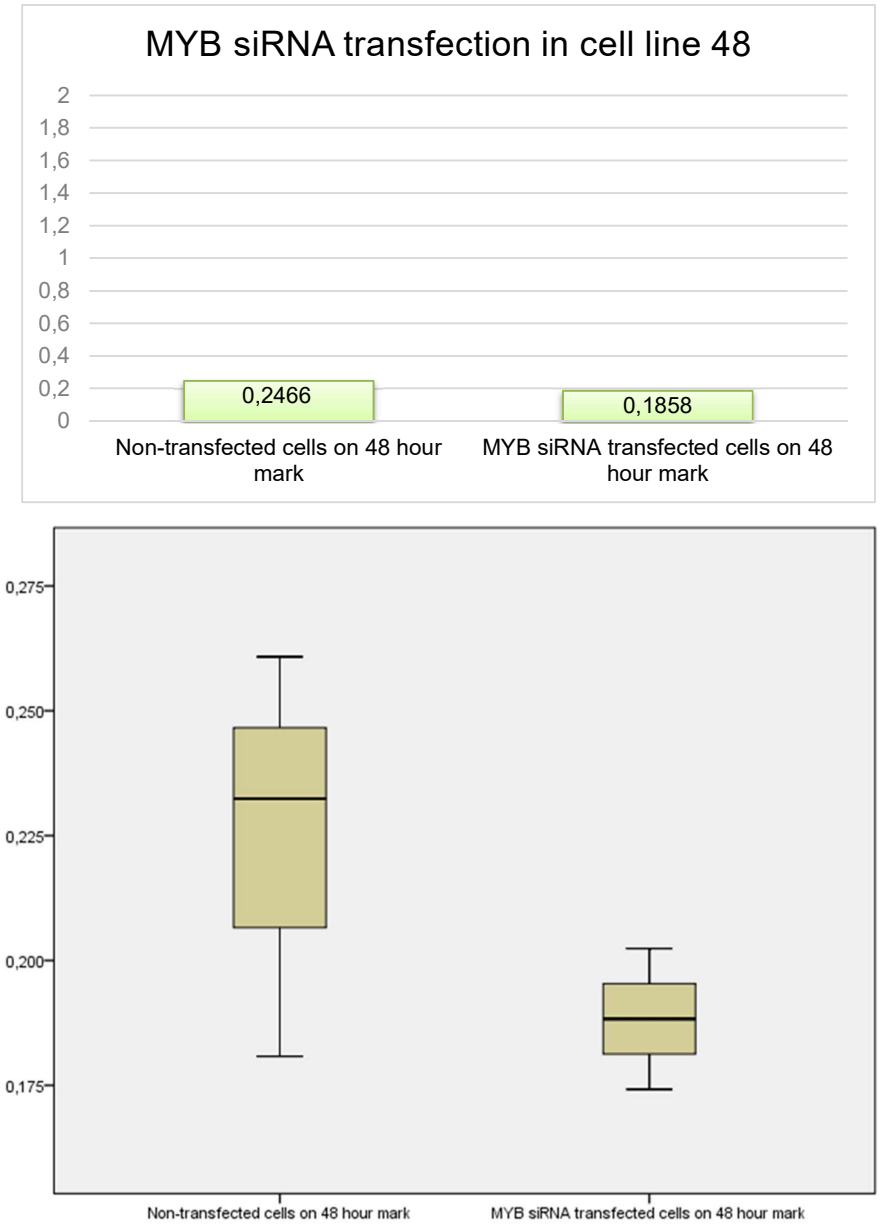
Picture 10. Validation graphic presentation for line 48 cells. For validation, before treatments and non-treated cells on 48 hour mark, were compared with each other. Mean values, of RNA result groups, were compared with each other. Validation is demonstrated as a histogram and as a box plot. Validation is not accurate, because the amount of MYB produced RNA is different in compared groups. Box plot has a different scale compared to histogram.

Between imatinib the non-treated cells on 48 hour mark and imatinib treated cells on 48 hour mark, there was a minor drop of *MYB* produced RNA level in line 48. However, the decreasing of *MYB* produced RNA in imatinib treated cells is extremely small or hardly recognisable. Hence, the imatinib drug did not have the hoped effect on cell line 48 neither on RNA level in qPCR nor on protein level in western blot. The 48 cells did not grown properly and were forced, for the reason of limited amount of time, to be lysed before optimal confluence. This might be the major reason why the results from line 48 are not coherent with the expected reaction. The effect of imatinib in line 48 cells is demonstrated in Picture 11. The effect of imatinib is represented as a histogram and as a box plot.



Picture 11. Graphic presentation of effect of imatinib for line 48 cells. Non-treated cells on 48 hour mark and imatinib treated cells on 48 hour mark, were compared with each other. The imatinib treatment decreased the amount of MYB produced RNA in extremely minor amount, practically not at all, from 0,2085 to 0,2064 RNA. Mean values, of RNA result groups, were compared with each other. Effect of imatinib to cell line 48 cells is demonstrated as a histogram and as a box plot. Box plot has a different scale compared to histogram.

Effect of MYB siRNA for line 48 cells was noticed by comparing non-transfected cells on 48 hour mark with transfected cells on 48 hour mark. There was a minor decreasing in *MYB* produced RNA in MYB siRNA transfected cells compared with non-transfected cells. Nevertheless, the decrease is quite small and therefore it is not significant. Results from qPCR in line 48 indicate the same as did the results from western blotting in line 48 cells. Due to difficulties met in the process of growing line 48 cells, especially for qPCR, noteworthy results were only gained from line 882 cells. Effect of MYB siRNA in cell line 48 is demonstrated in Picture 12. The effect is represented as a histogram and as a box plot.



Picture 12. Graphic presentation of effect of MYB siRNA for line 48 cells. Non-transfected cells on 48 hour mark and MYB siRNA transfected cells on 48 hour mark, were compared with each other. The MYB siRNA transfection decreased the amount of MYB produced RNA in minor amount from 0,2466 to 0,1858 RNA. Mean values, of RNA result groups, were compared with each other. Effect of MYB siRNA to cell line 48 cells is demonstrated as a histogram and as a box plot. Box plot has a different scale compared to histogram.

5 Discussion

In this study, cell culturing was based on aseptic work methods. GIST cells were cultured in suitable environment (RPMI base). Heat inactivated FBS and suitable antibiotics were added into the used medium of the cell culture. Cells were maintained by dividing them and by changing the medium on regular basis. To this cell growth environment, imatinib was added and incubated 24 or 48 hours to let it effect on the cells of the culture. Small interfering RNAs were transfected to the cells by using Lipofectamine 2000 reagent. After the transfection, cells were left for 48 hours before any further tests could be performed that the gene or protein expression had time to fade. Every step in this study was marked down in to the laboratory notes.

In the cell culture most of the used reagents and used materials were new and were not expired. Only the inhibitors used in RIPA buffer were expired in the year 2015. Culturing was performed by following aseptic regulations and in proper conditions for cell culturing. Cells or sterilized materials/reagents contacted with cells, were only handled in sterilized laminar flow hood with sterilized gloves and medium among other reagents was always warmed to +37 °C before use. Used cell lines were kept in -150 °C in DMSO reagent to retain their morphological and physiological features. DMSO was deactivated and washed with PBS before the actual transfer and starting of cell culture in flasks filled with growth medium. Cells were kept during their culture period in incubator cabinet at +37 °C with 5 % of carbon dioxide. The adding of imatinib and the siRNA transfections were performed following the given and planned instructions of the research team (working protocol in Appendix 2). The procedure of western blotting went according to the used protocol (seen in Appendix 3).

References were mainly used in this study for the background of GIST, *MYB* gene and for the theory of used methods. Most of the used references were from different research articles and from different books concerning laboratory methods. Some of the information in the used sources might have been out to date due to new research on the GIST and *MYB* field. Nevertheless, the information in the used articles was relevant to this study and mainly from this century. The information in the book sources was accurate in describing the laboratory techniques used in this study. Techniques were straight forward, well studied and formed to be so called basic techniques in modern laboratories. This study did not require any ethical permissions et cetera because the used materials did not require any patient obligation to maintain secrecy as no direct patient samples were

used, no animal testing included nor genetically modified organisms were included in this study. All the permissions needed to conduct this study were handled and possessed by the research group of Heikki Joensuu. However, some ethical concerns in this study might have been the problems of ‘creating’ or growing new cancer cells which was done in a controlled environment. Some environmentally harmful materials were used for example β-mercaptoethanol, but all these kind of materials were stored and disposed according to the given manufacturer’s instructions.

This being said, the protocol for this research took time to find and form to gain proper usable results. There was a lot of fixing in many parts such as finding the right lightning exposure time, right chemiluminescent substrates, loading the right amount of protein, right concentration for the used antibody et cetera.

There were notable differences between the two used cell lines. Firstly, the line 882 grew in the cell culture quite fast and were morphologically larger cells than line 48 cells. Secondly, the line 882 seemed to react less to changes than line 48. All throughout the process the line 882 was more manageable than the line 48. Line 48 had small cells that grew differently depending upon the surface, for example they grew better in big flasks rather than in 6- or 24-well plate. Also line 48 cells, were easily damaged by changes in environment. Especially in the used 24-well plate for qPCR cells should have been cultured in different plates rather than on the same plate. Merely this, because line 882 cells had to be lysed line 882 before the line 48 cell due to the slow growth of line 48 and due to the fast growth of 882. This being said, the cells from 48 were removed few times from the incubator. The harm in doing so can be witnessed in line 882 as it was almost impossible to get enough cells from the plate while trying not to damage line 48 cells at the same time. This can be seen in pictures 7 and 10 of qPCR validation graphic demonstration (the columns should be on the same level rather than the other one being smaller in numbers).

There might have been unknown contaminations, too many passages, unique cell behaviour or some other changes in growth that effected the line 48 not to grow properly. Some of the reasons might have contamination of mycoplasma, of virus or wrong atmosphere for line 48 cells to grow. There might be also some problems in transfection or reaction to the imatinib drug why the results from the line 48 are not the searched results.

Additionally, problems in cell culture may reflect from unexperienced working in cell culture. These are the main reasons why there are only proper usable results from line 882 for this study.

As mentioned earlier the used inhibitors for RIPA buffer were out of date during this study. Nevertheless, it did not seem to have any major effect on cell lysis from the surface of flasks or plates. Inhibitors prevent enzymes for example proteases form breaking the wanted proteins in cell lysis. Cells were lysed on an ice bath and overall lysed by following the given instructions of the research team (demonstrated in Appendix 2). This being said, it was humanly impossible to get every single cell off from the cell culture bases. Despite that, all those cells that could be removed were removed under the given circumstances. Unfortunately, this might be seen in results as too little cell material.

After lysis, the breaking of cell structures was performed by using the sonic prep machine that breaks the structures using high pitch sound waves. The breaking was done in parts for ten seconds two times per each lysed cell sample in Eppendorf tube. Cells for western blotting did not react badly to this and were able to be in the sonic machine for the wanted 10 seconds' time. Cells used for qPCR however reacted fast to the sound waves and turned white quite quickly. Turning white indicated the breaking of also the smaller parts for example proteins in cells, which was not the meant reaction when only the larger parts were meant to brake. This might have occurred because of the smaller amount of cells from the used 24-well plate instead of T25 flasks in the case of western blotting. For this reason, the preparation for qPCR cells was only few seconds per Eppendorf tube. This is why there maybe were not enough material to be isolated and that might be part of the reason why small amounts of RNA was used. More RNA might have given the way to get more samples and more triplicates from those samples for the qPCR, which would have given the opportunity for statistical analysis.

The lysed cells were kept in -70 °C and then thawed for used. Because of the search for the right protocol some lysed samples had to be thawed few times. This might have affected the samples (some unwanted biochemical reactions may have occurred during the process). Samples were nevertheless thawed on an ice bath to avoid any harmful biochemical reactions. To measure the amount of protein for western blotting, absorbance method in measuring the total protein amount was used. The samples were compared to the made protein standards provided by the used protein measurement kit (seen in Appendix 5). The measuring had to be performed few times because there were too

significant differences between duplicates in the measured samples. The difference originated from inaccurate pipetting. Approved and left results can be seen in Appendix 5. Due to these problems the approved results were not as accurate as wanted in total protein amounts (which would have been under 10% difference between duplicates). Overall, absorbance results told the round number of total protein in the lysed samples. These results were used in loading the samples in SDS-PAGE gels in western blotting.

β-mercaptoethanol was added to the lysed cells for denaturing the protein structures of the cells and the samples were boiled in +100 °C for ten minutes. After the denaturation, the samples were ready to be loaded in to the SDS-PAGE gel. Overall the denaturation and gel loading went according to the protocol. Neither there were any problems in gel electrophoresis nor in building the so called 'sandwich' for electroblotting the proteins from the gel into the PVDF membrane. Samples were thoroughly washed with washing buffer between antibodies and also profoundly kept in the used chemiluminescent. Used reagents and materials were up to date and freshly made for this procedure.

The problems laid in antibody concentrations and in the lightning time of used x-ray films. First the primary antibody was made to concentration 1:1000 that did not give a proper picture. Also the lightning was poor with the used chemiluminescent (one and a half hour lightning was not enough). Moreover, it was discovered that the MYB antibody allowed only one lightning period with the same chemiluminescent attached to the membrane. This is why the chemiluminescent had to be washed from the membranes first and then replace the used primary antibody with new one overnight and carry on from there over again. Since this protocol did not work, antibody concentration was increased to 1:500 and lightning time was added up to two to five hours. This protocol gave a slight bands which were interpreted that the MYB band could be found from the membranes. The bands were too light to say anything about the effects of imatinib or the MYB siRNA. For this reason, pico-chemiluminescent was replaced to the femto-chemiluminescent with 1:500 antibody and quick light exposure. The femto turned out to be extremely sensitive so the final results were gained adding 1:3 of femto in pico chemiluminescent, 1:500 primary antibody, 1:10 000 secondary antibody and 30 or 45 second lightning exposure. This complex gave the best results in given time for western blot pictures.

For qPCR the cells were cultured in 24-well plate. The first attempt to culture line 882 and 48 in 24-well plate did not succeed at all. The cells did not grow or they grew extremely little so we were unable to use them for the RNA isolation. On the second time,

line 882 grew on acceptable level, but the line 48 grew barely at all. Nevertheless, the cells were used for control and for imatinib treatment or MYB siRNA transfection. The before treatments controls were cultured on a different plate and that might have been the reason why they grew more firmly. Although, the line 882 cells were hard to remove from the plate. The difficulties emerged, because the line 882 had to be removed from the plate earlier than the line 48 (line 48 cells were not in a proper confluence for transmission). 882 cells had to be removed extremely carefully trying not to damage line 48 cells. This difficulty can be seen in validation graph of line 882 in Picture 7. There were not enough cells to gain the wanted validation results. Overall, the cell lines should have been cultured on different plates due to the fact that line 48 grew so much slower or hardly at all than the line 882. This fact had effect on both of the lines. It was unable to get line 882 cells enough to get better results and line 48 cells did not appreciate the moving around from the incubation storage. The cells were stored in -70 °C before the RNA isolation.

RNA isolation was performed according to Machery-Nagel's RNA silica isolation protocol. Due to mistake in washing part of the silica method, the RNA was washed multiple times to ensure the purity of RNA. Accidentally Buffer 3 was used raw instead of diluting it in to ethanol. The mistake was fixed by washing the cells multiply times with ethanol before diluting them into sterile RNAase free water. After the elution, the purity and concentration of RNA was measured by using the absorbance of 260nm. Purity of the RNA varied from 1,53 ng/µl to 2,12 ng/µl (seen in Appendix 5) and the yield of RNA in the actual samples (not in the control) varied from 2,5 to 23,5 (Appendix 5). After the isolation RNA was transferred to cDNA for qPCR. The amount of the used RNA template was determined by the yield of RNA. Under 10ng had 15µl of template and 60µl of reaction mix; from 10ng to 16,4ng had 7µl of template and 28µl reaction mix and 49µl sterile water; and lastly over 16,4ng had 12µl of template and 48 reaction mix and 132µl sterile water. The transfer from RNA to cDNA was performed with Dyad Disciple machine according to the given program (Appendix 4). After the transfer the samples were ready for quantitative PCR.

For qPCR the samples were divided with the control sample and with water control for three different 96-well plates (for *MYB* and *G6PD*). Overall there were 211 samples for the qPCR. The used *MYB* primers were in a 100 µM stock each and were diluted in a ratio 1:10. For each well 1µl of sample and 19µl of reaction mix was pipetted. All of the 96-well plates were pipetted and run during one day. For that reason, there is a possibility

for human errors in pipetting the samples and the reaction mix. Moreover, the possibility for human errors increases in small amounts of pipetting per one well (1µl of sample). The procedure was done according to aseptic working methods in a fume hood to avoid any contaminations of the samples. The plates were analysed in 1,5 hours per plate. In few triplicate samples the results were not usable for unknown reason in the working period. Those samples might had some contamination, not the right amount of sample in the plate, mistakes in calculation of the sample or there were so little of the sample to begin with that no proper results could've be gained (samples non-treated cells and MYB siRNA transfected in 48 and also siRNA control in line 882).

Overall, there might be too few results in numbers to say anything absolute certain about the effect of imatinib or MYB siRNA. In western blotting, two time points and duplicates though indicate clearly the effect of the drug imatinib in line 882. The diminish of the estimated MYB protein signal can be visually seen to fade in both time points (24 hours and 48 hours of imatinib treatment). This way the results gave an answer to one of the hypothesis of this study. On the other hand, in the qPCR it was not able to use any statistical methods due to the fact that there were few triplicates or duplicates used. To gain statistical proof of RNA level action or drop of the *MYB* coded mRNA, there should be larger quantity of samples to get statistically significant results. Two or three samples can only tell part of the truth about the RNA level effects on *MYB* in the case of imatinib or MYB siRNA.

This study succeeded well despite of all the difficulties met throughout this project. Main strengths – motivation, eagerness and interest in the subject – helped me concentrated in the actual working with the MYB antibody. Moreover, my quite short schedule compared to this project kept me concentrated all the time throughout this year. Generally well planned schedule and organizing time worked satisfactorily. There were minor mishaps in the planned schedule due to the difficulties of MYB antibody and due to other university of applied sciences or work projects that were simultaneous with this thesis. Overall I learned a lot about cancer research and working rhythm in a research group. Also I gained a lot of new knowledge about genes and their linkage in different cancers. All the previous knowledge from my degree programme in biomedical laboratory science was brought to use in this project. Additionally, I grew tremendously as a biomedical laboratory scientist due to this thesis.

Regardless of difficulties with the cell line 48 and special working conditions with Millipore MYB antibody, proper results were gained from this study. The results indicate that there is a meaning of *MYB* expression concerning gastrointestinal stroma tumours. It is also interesting that the drug imatinib is seen to decrease the protein and the RNA production of *MYB* in these cells. MYB siRNA had effect also in the GISTs cells despite the fact that siRNAs rarely transfect 100% to cells. Results were gained from cell line 882 concerning imatinib's effect decreasing the production MYB transfection factor. Results from used MYB siRNA in line 882 were bit difficult to interpret due to the lack of expected KIT siRNA reaction, but they do give some kind of protein and RNA decrease in the results. Moreover, the creation of a proper and working protocol for MYB antibody was made during this study. This way it can be used more effectually in the future research of *MYB* gene.

There are some things that affected this study that should or could have been done better. The used Millipore MYB antibody was not tested before it was tested in this study. This was the main reason that affected the schedule and took more time in the laboratory than expected. There were problems in the right concentration of antibody and the right lightning time of western pictures. The fact that MYB antibody had to be washed off before taking new pictures from the same membranes was a problem. Additionally, there were problems with the finding of right chemiluminescent for the western pictures. It took several weeks to form a proper working protocol to gain usable results, which for its own part took time from for example the qPCR. With more time there could have been more samples to qPCR to gain results that could have been analysed with statistical methods. Statistical analysis would have given more trustworthy results about RNA level *MYB* gene function in GIST cancer cells. Unnecessary time consuming things were also rerunning the protein measurements and the difference in speed of growth between line 882 and line 48. Moreover, the amount of samples in qPCR demanded lot of concentration but had to be performed during one day. If there would have been more time, there maybe would not have been mistakes or possible contaminations in some of the qPCR samples.

The diminish of MYB protein and RNA signal, especially in imatinib treated line 882 cells, indicate that imatinib has effect of *MYB* gene expression and that gene expression is seen in GIST cancer cells. Further meaning of this requires more research in the field of *MYB* linkage of GIST cancer and especially *MYB* linkage to for example *KIT* gene. When imatinib is known to effect *KIT* mutation positive GIST cells, does these results indicate that *MYB* might control *KIT* gene expression? Is *MYB* really the main super enhancer to

downstream genes in GIST such as *KIT*? In the research group of Heikki Joensuu next steps in *MYB* research are to find out the effect of imatinib in downstream genes of *MYB* for example *KIT*, *SLUG* and stem cell factor. Moreover, the research group is going to find the answers to other questions, such as does *MYB* positive tumour patients have worse survival prediction? Does *MYB* gene expression effect on imatinib response? Could GIST tumours be treated with inhibiting *MYB* expression? Answers to these questions need foundation for GIST patient-drug testing and further research on cell level for example to study effect of reducing protein signals of MYB and seeing how it affects other genes or on other genes expression in proliferation of cancer cells.

Gaining knowledge of the genetic origins of cancer can be the foundation of genetically targeted cancer treatments. *MYB* could be one of the possible targets for personalized cancer treatments. Hopefully, this thesis could work as a part of targeting *MYB* in the future. Naturally, more research around *MYB* subject must be conducted before any treatments can be verified. In near future, these genetically targeting treatments might be seen playing a significant role in different cancer therapies.

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Reagents and materials

Cell culture medium

Product	REF/ Catalogue number	LOT	Exp.	Storage
Gibco – RPMI Medium	31870-025	1737520	2017/12	+4 °C
Gibco – Penicillin Streptomycin Glutamine	10378-016	1677652	2016/02	-20 to +5 °C
Gibco – Fetal Bovine Serum: inactivated at +56 °C	10270-106	41Q6841K	2019/09	-20 to +5 °C

Cell culture

Product	REF/ Catalogue number	LOT	Exp.	Storage
Lonza - Trypsin x10	BE02-007E	4MB146	2018/09	< -10 °C
Sigma - DMSO (Dimethyl sulphoxide)	D2650	RNBC9661	2016/07	Room temp.
Thermo Fisher Scientific – Pierce RIPA buffer	89901	QH220396	Not known	+4 °C
Thermo Fisher Scientific – Pierce Phosphatase Inhibitor	88667	OF189628	2015/05	+4 °C

Thermo Scientific – Pierce Protease inhibitor: AEBSF, E&¤, Leupeptin, Aprotinin, Pepstatin A and Bestatin	88666	OJ194838A	2015/01	+4 °C
Gibco – Opti-MEM: Reduced Serum Medium	31985-047	1722740	2016/10	+2 to +8 °C
Invitrogen - Lipofectamine Invitrogen Reagent	11668-019	1692389	2017/05	+2 to +8 °C
Gibco – L-Glutamine 200mM	25030-024			-20 °C
Dharmacon – ON-TARGET plus Control pool Non-Targeting pool	D-001810-05	1993609	opened 020316	-20 °C
Dharmacon - ON-TARGET plus SMART pool Human MYB	L-003910-00	150708	opened 310815	-20 °C
Dharmacon – ON-TARGET plus SMART pool Human KIT	L-003150-00	160121	opened 020316	-20 °C

Gibco – Trypan Blue Stain (0.4%)	15250-061	1756514	2018/12	Room temp.
Thermo Scientific – Easy Flask 75 Filt		145060	2020/08	Room temp.
Cellstar – 6 Well Cell Culture Plate sterile with lid	657 160	E14103J7	2018/10	Room temp.
Costar – 24 Well Cell Culture Cluster: Flat Bottom with Lid: Nonpyrogenic: Polystyrene				
Lancrix – Imatinib mesylate 99%+	147881	130305	Not Known	Cool and dry
Amresco – Water, Sequencing grade	K683-25ml	1104C176		Room temp.

Western blotting

Product	**REF/ Catalogue number**	**LOT**	**Exp.**	**Storage**
Thermo Scientific – Pierce BCA Protein Assay Kit: Includes reagent A and B and also Albumin	Reagent A: 23228 Reagent B: 1859078 Standard: 23209	P1208796	Not Known	Room temp.

standards from bovine serum				
Bio Rad – 2x Laemmli Sample Buffer	161-0737	L004126A	2017/10	Room temp.
Sigma chemicals – β-mercaptoethanol C2H2OS	M-3148	95H092315		Room temp.
Bio Rad – 1x SDS buffer: Diluted 10x TRIS/Glycine/SDS-Buffer	161-0732	Not known	2015/11	Room temp.
1x Transfer Buffer: Diluted 10xTBS, tween added. 24,2q Tris Base, 80,0 g NaCl and 13,5 ml 37% HCl in 1 liter of MQ H2O				
Bio Rad – Mini-Protean TGX Precast Gels: 10%, 10-well-comb, 50 µl/well	456-1034		2016/10	+2 to +8 °C
BioRad – Precision Plus Protein Dual Color Standards (Ladder)	161-0374	ctrl 64022882	2017/09	-20 °C

Temecula - Jurkat Cell Lysate (T-Lymphocyte cell line)		2500604		-20 °C
Fisher Chemical – Methanol Analytical reagent grade		1666182	Packing date: 270116: Use within 5 years of opening	Room temp.
Millipore – Anti-Myb, clone 1-1: Monoclonal antibody	05-175	2605462		-20 °C
Jackson Immuno Research – Peroxide-conjugated Affinipure Rabbit Anti-Mouse IgG	315-035-003	65710		-70 °C
Valio – Rasvaton maitojauhe				
Sigma – Ponceau S Solution 0.1%: Ponceau S (w/v) in 5% acetic acid (v/v)	025K4355		Opened: 21.9.2005	Room temp.
Thermo Fisher Scientific – Supersignal West Pico Chemiluminescent	34080	QH220527	Not Known	Room temp.

Subsrate: Contains: Stable peroxide and Luminol/ Enhancer				
Thermo Fisher Scientific – Supersignal West Femto Maximum Sensitivity Substrate	34095	QG21978413	Not Known	Stable in room temperature for 6 months. Longer storage in +4 °C
Thermo Scientific – Nuclon Delta Surface 96 well plate Sterile	167008	145551	Not Known	Room temp.
Bethyl – Rabbit anti-Cytoskeletal Actin Affinity Purified: 100ul at 1mg/ml	A300-491A			+2 to +8 °C
Jackson Immuno Research – Peroxide-conjugated Affinipure Goat Anti-Rabbit IgG	111-035-003	108536		-70 °C

RNA protocol

Machery-Nagel – NucleoSpin RNA	740955.50	1506/006	Not known	Room temp.
Roche - Light Cycler 480 Probes Master (Probe mix and H2O for qPCR)	04887301001	Not known	Not known	Store at -15 to -25 °C
Roche – Universal ProbeLibrary Human G6PD Gene Assay: Probe	05046246001	091824	2015/06	Store in dark at -15 to -25 °C
Roche – Universal ProbeLibrary Human G6PD Gene Assay: Primer	050462466001	100180	2016/08	Store at -15 to -25 °C
Roche – Universal ProbeLibrary: Probe #54	0468851001	701237		Store at -15 to -25 °C
Oligomer – MYB_LNA#54_Forward primer	160323B067B11			Store at -15 to -25 °C
Oligomer – MYB_LNA#54_Reverse primer	160323B067C11			Store at -15 to -25 °C
Bio-RAD – iScript cDNA Synthesis Kit	170-8891	ctrl 64063853	2018/01	-20 °C

Working protocol for cell culture

By research group of Heikki Joensuu

Cells from freezer to flasks

1. Divide medium to two T75 flasks 15ml per each flask. Place them in to the incubator +37 °C and 5% CO2 to settle for 15 minutes.
2. Pick the cells from -150 °C and melt the suspension in Eppendorf tube quickly in warm water +37 °C.
3. Add 5 ml of medium to the cell suspension in falcon tube. Make sure that all the cells from the Eppendorf tube are mixed with the medium.
4. Centrifuge the falcon for 7 minutes in 1000 rpm.
5. Remove the medium from the cells and add 1 ml medium on top of the cells. Mix the cells and 1 ml medium carefully by pipetting few times.
6. Take the T75 flasks from the incubator and add 500 µl cell suspension per flask.
7. Place them back in to the incubator and monitor after few days how the cells have started to grow.

Dividing cells

1. Cells should be at least in 70 to 80 % confluence before dividing them in microscope.
2. Remove the medium from cells and wash them with 10ml PBS solution.
3. Remove the PBS and add trypsin on top of the cells, e.g. 2ml trypsin to GIST48 and 3ml trypsin to GIST882.
4. Place the flasks in to the incubator for 5 to 10 minutes.
5. Take the flasks out and tap the sides of flasks a bit to remove the cells from the surface of the flasks.
6. Add medium to the cells so that the total volume in flask is 10 ml. Rinse the surface of the flask with the medium and remove the suspension with trypsin and medium in to falcon tube.
7. Centrifuge the falcon for 7 minutes in 1000 rpm.
8. Remove the media from the falcon and add 1 ml of medium on top of the cells. Mix the cells and 1 ml medium carefully by pipetting few times.

9. Divide the cells into new flasks with 15 ml of medium, e.g. 500 µl in 1:2 or 333 µl in 1:3. If divided to smaller T25 flasks, use 5 ml of medium per flask (2 ml medium in 6 microtiter plate and 500 µl medium in 24 microtiter plate).
10. Place flasks into the incubator.

Imatinib treatment to cell culture

1. Prepare wanted concentration for imatinib by diluting it to the used RPMI medium from 10mM stock into 0,5µM (2,5 µl stock and 50ml of medium).
2. For the control cells change medium normally to the T25-flasks (5ml).
3. For the imatinib treated cells change medium by using the prepared 0,5µM imatinib medium (5ml).
4. Place the flasks back into the incubator.

siRNA transfection to cell culture

Lipofectamine™ 2000 Transfection Reagent (Invitrogen) 6-well plates

1. (DAY 0) Seed cells in 2 ml antibiotics-free growth medium. Cells should be ~30-50% confluent before starting transfections.
2. (DAY 1) Remove growth medium.
3. Wash the cells with 2ml PBS.
4. Add 2 ml of Opti-MEM medium to the cells and let the cells starve ~6 h.
5. Take the siRNAs on the ice.
6. Mix Lipofectamine before use by pipetting. Dilute 2,5 µl Lipofetamine in 250 µl Opti-MEM. Mix by pipetting.
7. Mix siRNAs before use by pipetting gently. Dilute 5 µl siRNA (20 µM stock) in 250 µl Opti-MEM
8. Incubate 5 min at room temp.
9. Add diluted Lipofectamine to diluted siRNA. Mix by pipetting ~5 times.
10. Incubate Lipofectamine-siRNA complex 20 min at room temperature.
11. Remove Opti-MEM from cells.
12. Add 1ml of antibiotics-free growth medium to cells.
13. Add Lipofectamine-siRNA complex (500 µl) to the cells.
14. Incubate cells 24 h at+37°C.
15. (DAY 2) Incubate cells 24-72 h at+37 °C.

16. (DAY 3-5) Visualize/analyze transfected cells.

Reactions are for one well

Lipofectamine™ 2000 Transfection Reagent (Invitrogen) T25 flask

1. (DAY 0)Seed cells in 5 ml antibiotics-free growth medium. Cells should be ~30-50% confluent before starting transfections.
2. (DAY 1)Remove growth medium.
3. Wash the cells with PBS.
4. Add 3 ml of Opti-MEM medium to the cells and let the cells starve ~6 h.
5. Take the siRNAs on the ice.
6. Mix Lipofectamine before use by pipetting. Dilute 20 µl Lipofetamine in 2ml Opti-MEM. Mix by pipetting.
7. Mix siRNAs before use by pipetting gently. Dilute 40 µl siRNA (20 µM stock) in 2ml Opti-MEM
8. Incubate 5 min at room temp.
9. Add diluted Lipofectamine to diluted siRNA. Mix by pipetting ~5 times.
10. Incubate Lipofectamine-siRNA complex 20 min at room temperature.
11. Remove Opti-MEM from cells.
12. Add 2 ml of antibiotics-free growth medium to cells.
13. Add Lipofectamine-siRNA complex (1 ml) to the cells.
14. Incubate cells 24 h at+37°C.
15. (DAY 2) Incubate cells 24-72 h at+37°C.
16. (DAY 3-5) Visualize/analyze transfected cells.

Reactions are for one well

Lipofectamine™ 2000 Transfection Reagent (Invitrogen) 24-well plates (for 1 well)

1. (DAY 0) Seed cells in 500µl of growth medium without antibiotics. Cells should be ~70-90% confluent before starting transfections.
2. (DAY 1)Remove growth medium.
3. Wash the cells with PBS.
4. Add 500µl of Opti-MEM medium to the cells and let the cells starve ~6 h.
5. Take the siRNAs on the ice.

6. Mix Lipofectamine before use by pipetting. Dilute 1,0 µl Lipofetamine in 50 µl Opti-MEM. Mix by pipetting gently.
7. Mix siRNAs before use by pipetting gently. Dilute 1,0 µl siRNA (20 µM stock) in 50 µl Opti-MEM
8. Add diluted Lipofectamine to diluted siRNA. Mix by pipetting ~5 times and incubate 5 min at room temperature. Proceed the step 7 in 25 minutes!
9. Add Lipofectamine-siRNA complex (100 µl) to the cells and mix gently by rocking the plate.
10. Incubate cells 24 h at+37°C.
11. (DAY 2) Replace Opti-MEM medium with normal growth medium including antibiotics.
12. Incubate cells 24-72 h at+37°C.
13. (DAY 3-5) Visualize/analyze transfected cells.

    If the MYB expression is not absent in any time points, siRNA concentration has to be optimized next.

Cell lysis

Procedure for Lysis of Monolayer-cultured Mammalian Cells
Note: If desired, add protease and phosphatase inhibitors to the RIPA Buffer immediately before use. (Pierce Protease and Phosphatase Inhibitor Mini Tablets, EDTA Free 1 tablet per 10 ml RIPA)

1. Carefully remove culture medium from adherent cells.
2. Wash cells once with PBS.
3. Add cold RIPA Buffer to the cells. Use 1ml of buffer per 75cm2 flask containing 5 000 000 HeLa or A431 cells. Keep on ice for 5 minutes, swirling the plate occasionally for uniform spreading.
4. Gather the lysate to one side using a cell scraper and collect the lysate into an Eppendorf tube.
5. Sonicate the pellets for 10 seconds per tube.

Working protocol for western blotting

By research group of Heikki Joensuu

Procedure for Lysis of Monolayer-cultured Mammalian Cells

Note: If desired, add protease and phosphatase inhibitors to the RIPA Buffer immediately before use. (Pierce Protease and Phosphatase Inhibitor Mini Tablets, EDTA Free 1 tablet per 10 ml RIPA)

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4. Gather the lysate to one side using a cell scraper and collect the lysate into an Eppendorf tube.

   Note: To increase yields, sonicate the pellet for 10 seconds with 50% pulse.

5. Transfer supernatant to a new tube for further analysis.

Measure the protein concentrations with Pierce BCA protein assay KIT
Mix the sample with LAEMMLI buffer (1/1)

SDS-PAGE

Carry out SDS-PAGE (10% Mini-PROTEAN® TGX™ Gel, 10 well)
For analytical runs, load 25 to 50 micrograms of protein per well. Apply constant voltage at 100 V for max 1.5 hours.
Running Buffer: Bio-Rad Tris/Glycine/SDS buffer (dilute 10x buffer for 1x buffer)
Ladder: 4µl Bio-Rad Dual colour ladder.

Protein Blotting

1. Build the transfer "sandwich" onto the bright side of cassette as follows: 1 sheet foam pad, 1 sheet filter paper, PVDF membrane, gel, 1 sheet filter paper, 1 sheet foam pad
2. Carry out the transfer at 0.15 A overnight at +4°C.
3. Remove the PVDF membrane from the apparatus (and check the transfer efficiency with Ponceu S).

NOTE: Prewet the PVDF membrane in methanol before it is used (1min).
NOTE: Prewet the all other membranes in transfer buffer before making the “sandwich”.

Immuno-Detection

1. Block PVDF blot using 5% w/v milk (or BSA) in TBS-Tween 1 h at room temperature. The choice of blocking reagent depends on the type of antibody that will be subsequently used in the overlay procedure and should be chosen accordingly.
2. Remove the blocking buffer.
3. Wash the blot 3 times for 5 min with TBS-T.

4. Overlay the blot with 20 ml primary antibody at an appropriate dilution (Millipore MYB ab: 1% milk 1:1000/ 1:500).
5. Incubate overnight at +4°C on shaker.
6. Wash the blot three times for 5 minutes each, with sufficient TBS-T.
7. Incubate the blot for 1 hour in Rabbit Anti-Mouse IgG -secondary antibody conjugate at an appropriate dilution. (1:10 000) in 1% milk).
8. Wash the blot three times for 5 minutes each, with sufficient TBS-T.
9. Prepare SuperSignal™ West Pico Chemiluminescent Substrate Working Solution by mixing equal parts of the Stable Peroxide Solution and the Luminol/Enhancer Solution (5ml+5ml). Mix it with prepared (1:1) SuperSignal™ West Femto Maximum Sensitivity Substrate Working Solution on ratio 1:3 (femto 1:3 and pico 2:3).
10. Incubate the blot in the substrate mixture for 5 minutes.

11. Remove blot from Working Solution and place it in a plastic membrane protector; a plastic sheet protector or plastic wrap may be used. Place the protected membrane in a film cassette with the protein side facing up.

Working protocol for quantitative PCR

By research group of Heikki Joensuu

RNA isolation

RNA isolation was performed according to Machery-Nagel user manual of RNA isolation version User manual NucleoSpin® RNA XS 2014.

RNA transfer to cDNA

1. Measure the purity and concentration of isolated RNA by using e.g. spectrophotometer.
2. Prepared iScript cDNA mix and sterile water are combined with the RNA template sample.

   For 1x reaction

5x iScript	4µl
iScript transcriptase	1µl
Sterile water	x µl
RNA template	x µl
Total volume	20µl

3. Samples are transferred in Thermal Cycler: Dyad Disciple – machine on selected program.

   Program:
   Incubation +25 for 5 min
   Incubation +46 for 20min
   Incubation +95 for 1min
   Incubation +4
   Ending

qPCR

Prepare PCR mixes for MYB gene and for control gene G6PD
(For 211 samples)

MYB gene PCR mix

- Probe buffer 1100 µl
- Forward primer (10 µM) 44 µl
- Reverse primer (10 µM) 44 µl
- Probe 22 µl
- Sterile water 880 µl

G6PD gene PCR mix

- Probe buffer 1100 µl
- Primer mix 44 µl
- Probe 44 µl
- Sterile water 902 µl

Pipette on 96-well plate 1µl of sample and 19µl of PCR mix to each well
Program the Light Cycler 480 for 55 cycles (duration round 1,5 hours)

Charts

Used amounts of RIPA buffer in cell lysis

	Sample	Confluence	RIPA
T25 flasks imatinib treatment after 24 hours	GIST 48 non-treated	60 %	200 µl
	GIST 48 imatinib	20 %	100 µl
	GIST 882 non-treated	100 %	400 µl
	GIST 882 imatinib	80 %	300 µl
T25 flasks imatinib treatment after 48 hours	GIST 48 non-treated	80 %	200 µl
	GIST 48 imatinib	10 %	100 µl
	GIST 882 non-treated	100 %	400 µl
	GIST 882 imatinib	70 – 80 %	300 µl
6-well plate siRNA transfection line 882 after 48 hours (*not in the final results)	GIST 882 negative control	Not marked down in laboratory notes	100 µl
	GIST 882 MYB siRNA	Not marked down in laboratory notes	100 µl
	GIST 882 KIT siRNA	Not marked down in laboratory notes	100 µl
6-well plate siRNA transfection line 48 after 48 hours (*not in the final results)	GIST 48 negative control	Not marked down in laboratory notes	50 µl
	GIST 48 MYB siRNA	Not marked down in laboratory notes	50 µl
	GIST 48 KIT siRNA	Not marked down in laboratory notes	50 µl
T25 flask siRNA transfection line 882 after 48 hours	GIST 882 negative control	90 – 100 %	500 µl
	GIST 882 MYB siRNA	80 – 90 %	500 µl
	GIST 882 KIT siRNA	80 – 90 %	500 µl

T25 flask siRNA transfection line 48 after 48 hours	GIST 48 negative control	90 – 100 %	500 µl
	GIST 48 MYB siRNA	90 – 100 %	500 µl
	GIST 48 KIT siRNA	100 %	500 µl

*6-well plate siRNA transfected cells were used at the beginning of the proses. Because of the difficulties of MYB antibody cell lysates ran out, new siRNA transfected cells were cultured in T25 flasks to get more lysate for western blotting.

Protein BCA absorbance measurements for western blotting

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Ke 13.4.2016 IMATINIB

Session: C:\ASCSW26\NANNA.SEE
Instrument: MULTISKAN EX PRIMARY EIA V. 2.3
User name: Nanna
Started at: 4/13/2016 2:9:38PM
Actual temperature: Amb.temp.
Comment:

Layout map for calibrators Sheet: Blank1, Assay: Assay1 and for samples Sheet: Blank1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Cal_8	Cal_8	882 D2 IM2	882 D2 IM2	48 D2 IM2	48 D2 IM2				
B	Cal_1	Cal_1	882 D1 ctrl	882 D1 ctrl	48 D1 ctrl1	48 D1 ctrl1						
C	Cal_2	Cal_2	882 D1 ctrl	882 D1 ctrl	48 D1 ctrl2	48 D1 ctrl2						
D	Cal_3	Cal_3	882 D1 IM1	882 D1 IM1	48 D1 IM1	48 D1 IM1						
E	Cal_4	Cal_4	882 D1 IM2	882 D1 IM2	48 D1 IM2	48 D1 IM2						
F	Cal_5	Cal_5	882 D2 ctrl	882 D2 ctrl	48 D2 ctrl1	48 D2 ctrl1						
G	Cal_6	Cal_6	882 D2 ctrl	882 D2 ctrl	48 D2 ctrl2	48 D2 ctrl2						
H	Cal_7	Cal_7	882 D2 IM1	882 D2 IM1	48 D2 IM1	48 D2 IM1						

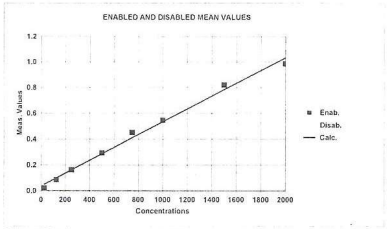
Source data for calibrators Sheet: Blank1, Assay: Assay1 and for samples Sheet: Blank1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.003	-0.002	0.964	1.006	0.147	0.142	0.395	0.418				
B	0.020	0.020	0.226	0.219	0.514	0.560						
C	0.082	0.087	0.254	0.216	0.511	0.619						
D	0.143	0.180	0.163	0.175	0.467	0.438						
E	0.291	0.294	0.188	0.191	0.319	0.376						
F	0.423	0.478	0.540	0.257	0.607	0.601						
G	0.551	0.540	0.272	0.258	0.668	0.508						
H	0.821	0.818	0.190	0.166	0.375	0.407						

Sheet: Blank1, Assay: Assay1

Name	Meas.	Conc.
Cal_1	0.020	
	0.020	
	0.020	25.000
Cal_2	0.082	
	0.087	
	0.085	125.000
Cal_3	0.143	
	0.180	
	0.161	250.000
Cal_4	0.291	
	0.294	
	0.292	500.000
Cal_5	0.423	
	0.478	
	0.450	750.000
Cal_6	0.551	
	0.540	
	0.545	1000.000
Cal_7	0.821	
	0.818	
	0.820	1500.000
Cal_8	0.964	
	1.006	
	0.985	2000.000

	Conc.	Meas.	CalcConc.	Residual
Cal_1	25.000	0.020	-35.299	60.299
Cal_2	125.000	0.085	95.359	29.641
Cal_3	250.000	0.161	249.134	0.866
Cal_4	500.000	0.292	512.460	-12.460
Cal_5	750.000	0.450	830.059	-80.059
Cal_6	1000.000	0.545	1021.020	-21.020
Cal_7	1500.000	0.820	1572.799	-72.799
Cal_8	2000.000	0.985	1904.469	95.531



Mean Blank 0.119
Status:
Remark:
Fit type: Linear regression (SVD): y = a+b*x
Meas. transformation: Linear
Conc. transformation: Linear
Parameters: a 0.037, b 0.000
Corr. coeff. R2: 0.985

Calculated concentrations Sheet: Blank1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10	11	12
A	<min	<min	1862.256	>max	2199.868	2099.362	1436.994	1529.460				
B	-35.299	-35.299	3787.864	3647.156	1915.403	2100.334						
C	89.329	101.390	4350.698	3586.852	1903.342	2341.549						
D	211.946	286.321	2521.488	2762.702	1726.451	1609.864						
E	509.444	515.475	3024.018	3084.322	1131.455	1360.609						
F	774.780	885.337	1.01E+04	4411.002	2293.306	2265.164						
G	1032.076	1009.965	4712.520	4431.103	2534.520	1891.281						
H	1576.819	1568.778	3064.221	2581.791	1356.589	1485.237						

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Calibrators and samples Sheet: Blank1, Assay: Assay1

Name	Meas.	Calc.	Dil. factor	Result	SD	CV-%	Comment	Comment 2
Blank	0.003	<min	1.0	**#VALUE!**				
	-0.002	<min	1.0	**#VALUE!**				
	6.94E-18			**#VALUE!**	#VALUE!	#VALUE!		
Cal_1	0.020	-35.299	1.0	**-35.30**				
	0.020	-35.299	1.0	**-35.30**				
	0.020			**-35.30**	0	0		
Cal_2	0.082	89.329	1.0	**89.33**				
	0.087	101.390	1.0	**101.39**				
	0.085			**95.36**	8.528	8.94		
Cal_3	0.143	211.946	1.0	**211.95**				
	0.180	286.321	1.0	**286.32**				
	0.161			**249.13**	52.591	21.11		
Cal_4	0.291	509.444	1.0	**509.44**				
	0.294	515.475	1.0	**515.47**				
	0.292			**512.46**	4.264	0.83		
Cal_5	0.423	774.780	1.0	**774.78**				
	0.478	885.337	1.0	**885.34**				
	0.450			**830.06**	78.175	9.42		
Cal_6	0.551	1032.076	1.0	**1032.08**				
	0.540	1009.965	1.0	**1009.96**				
	0.545			**1021.02**	15.635	1.53		
Cal_7	0.821	1576.819	1.0	**1576.82**				
	0.818	1568.778	1.0	**1568.78**				
	0.820			**1572.80**	5.685	0.36		
Cal_8	0.964	1862.256	1.0	**1862.26**				
	1.006	>max	1.0	**#VALUE!**				
	0.985			**#VALUE!**	#VALUE!	#VALUE!	25/	
48 D1 ctrl1	0.514	957.701	2.0	**1915.40**			200787	
	0.560	1050.167	2.0	**2100.33**				
	0.537			**2007.87**	130.766	6.51	= vastaus x 2	
48 D1 ctrl2	0.511	951.671	2.0	**1903.34**			= 24,9 µl	
	0.619	1170.774	2.0	**2341.55**				
	0.565			**2122.45**	309.859	14.60	≈ 23,6 µl	
48 D1 IM1	0.467	863.226	2.0	**1726.45**				
	0.438	804.932	2.0	**1609.86**				
	0.452			**1668.16**	82.439	4.94	≈ 30 µl	
48 D1 IM2	0.319	565.728	2.0	**1131.46**				
	0.376	680.305	2.0	**1360.61**				
	0.347			**1246.03**	162.036	13.00	≈ 40,1 µl	
48 D2 ctrl1	0.607	1146.653	2.0	**2293.31**				
	0.601	1132.582	2.0	**2265.16**				
	0.604			**2279.23**	19.899	0.87	≈ 21,9 µl	
48 D2 ctrl2	0.668	1267.260	2.0	**2534.52**				
	0.508	945.641	2.0	**1891.28**				
	0.588			**2212.90**	454.839	20.55	≈ 22,6 µl	
48 D2 IM2	0.395	718.497	2.0	**1436.99**				
	0.418	764.730	2.0	**1529.46**				
	0.406			**1483.23**	65.383	4.41	≈ 33,7 µl	
48 D2 IM1	0.375	678.295	2.0	**1356.59**				
	0.407	742.618	2.0	**1485.24**				
	0.391			**1420.91**	90.968	6.40	≈ 35,2 µl	
882 D1 ctrl1	0.226	378.786	10.0	**3787.86**				
	0.219	364.716	10.0	**3647.16**				
	0.222			**3717.51**	99.496	2.68	≈ 26,9 µl	

väärä laimennus-kerroin 1:5 :2 = 1858,575

882 D1 ctrl2	0.254	435.070	10.0	4350.70			
	0.216	358.685	10.0	3586.85			
	0.235			3968.78	540.121	13.61	1984,39 ≈ 25,2 µl
882 D1 IM1	0.163	252.149	10.0	2521.49			
	0.175	276.270	10.0	2762.70			
	0.169			2642.10	170.564	6.46	1321,05 ≈ 37,8 µl
882 D1 IM2	0.188	302.402	10.0	3024.02			
	0.191	308.432	10.0	3084.32			
	0.189			3054.17	42.641	1.40	1527,085 ≈ 32,7 µl
882 D2 ctrl1	0.540	1009.965	10.0	1.01E+04			
	0.257	441.100	10.0	4411.00			
	0.398			7255.32	4022.479	55.44	2205,5 ≈ 22,7 µl
882 D2 ctrl2	0.272	471.252	10.0	4712.52			
	0.258	443.110	10.0	4431.10			
	0.265			4571.81	198.992	4.35	2285,905 ≈ 21,9 µl
882 D2 IM2	0.147	219.987	10.0	2199.87			
	0.142	209.936	10.0	2099.36			
	0.144			2149.62	71.069	3.31	1074,81 ≈ 46,5 µl
882 D2 IM1	0.190	306.422	10.0	3064.22			
	0.166	258.179	10.0	2581.79			
	0.178			2823.01	341.129	12.08	1477,505 ≈ 35,4 µl

- pienimmän mukaan lasketaan eli 1074,81 ng (menee eniten)
- 10 µg per kaivo (yleensä)
- 10 : 1,07481 = 9,30397
- koska näytteet laimennettu 1:2

9,30397 × 2 = 18,60794
≈ 18,6 µl

⇒ 25 µg per kaivo proteiinia

Ascent Software — Curve Fit1 — 5/12/2016 2:58 PM

3. mittaus 120576 70µl ⇒ TÄSTÄ LASKUT

10µl tuoppa 130516 uusinta ⇒ ei onnistunut

Session: C:\ASCSW26\NANNA.SEE
Instrument: MULTISKAN EX PRIMARY EIA V. 2.3
User name: Nanna
Started at: 5/12/2016 2:57:27PM
Actual temperature: Amb.temp.
Comment: siRNA T25

Layout map for calibrators Sheet: Blank1, Assay: Assay1 and for samples Sheet: Blank1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Cal_8	Cal_8	48 sictrl2	48 sictrl2						
B	Cal_1	Cal_1	882 sictrl1	882 sictrl1	48 siMYB1	48 siMYB1						
C	Cal_2	Cal_2	882 sictrl2	882 sictrl2	48 siMYB2	48 siMYB2						
D	Cal_3	Cal_3	882 siMYB	882 siMYB	48 siKIT1	48 siKIT1						
E	Cal_4	Cal_4	882 siMYB	882 siMYB	48 siKIT2	48 siKIT2						
F	Cal_5	Cal_5	882 siKIT1	882 siKIT1								
G	Cal_6	Cal_6	882 siKIT2	882 siKIT2								
H	Cal_7	Cal_7	48 sictrl1	48 sictrl1								

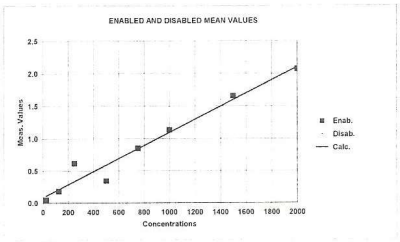
Source data for calibrators Sheet: Blank1, Assay: Assay1 and for samples Sheet: Blank1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	2.035	2.103	0.312	0.373						
B	0.044	0.046	0.391	0.145	0.307	0.282						
C	0.190	0.179	0.440	0.203	0.271	0.272						
D	0.345	0.342	0.296	0.275	0.273	0.262						
E	0.614	0.615	0.236	0.206	0.272	0.250						
F	0.862	0.839	0.210	0.214								
G	1.145	1.121	0.228	0.220								
H	1.667	1.645	0.264	0.260								

Sheet: Blank1, Assay: Assay1

Name	Meas.	Conc.
Cal_1	0.044	
	0.046	
	0.045	25.000
Cal_2	0.190	
	0.179	
	0.185	125.000
Cal_3	0.345	
	0.342	
	0.344	500.000
Cal_4	0.614	
	0.615	
	0.615	250.000
Cal_5	0.862	
	0.839	
	0.851	750.000
Cal_6	1.145	
	1.121	
	1.133	1000.000
Cal_7	1.667	
	1.645	
	1.656	1500.000
Cal_8	2.035	
	2.103	
	2.069	2000.000

	Conc.	Meas.	CalcConc.	Residual
Cal_1	25.000	0.045	-41.673	66.673
Cal_2	125.000	0.185	96.704	28.296
Cal_3	500.000	0.344	254.424	245.576
Cal_4	250.000	0.615	523.242	-273.242
Cal_5	750.000	0.851	757.343	-7.343
Cal_6	1000.000	1.133	1037.569	-37.569
Cal_7	1500.000	1.656	1556.359	-56.359
Cal_8	2000.000	2.069	1966.034	33.966



Mean Blank 0.121
Status:
Remark:
Fit type: Linear regression (SVD): y = a+b*x
Meas. transformation: Linear
Conc. transformation: Linear
Parameters: a 0.087, b 0.001
Corr. coeff. R2: 0.920

Calculated concentrations Sheet: Blank1, Assay: Assay1

	1	2	3	4	5	6	7	8	9	10	11	12
A	<min	<min	1932.308	>max	2231.773	2836.863						
B	<min	-40.681	3015.414	575.216	2182.176	1934.188						
C	102.159	91.248	3501.470	1150.547	1825.074	1834.993						
D	255.912	252.936	2073.061	1864.752	1844.913	1735.798						
E	522.746	523.738	1477.891	1180.306	1834.993	1616.764						
F	768.750	745.935	1219.984	1259.662								

G	1049.472	1025.665	1398.535	1319.179
H	1567.270	1545.447	1755.637	1715.959

Calibrators and samples Sheet: Blank1, Assay: Assay1

Name	Meas.	Calc.	Dil. factor	Result	SD	CV-%	Comment	Comment 2
Blank	0	<min	1.0	#VALUE!				
	0	<min	1.0	#VALUE!				
	0			#VALUE!	#VALUE!	#VALUE!		
Cal_1	0.044	<min	1.0	#VALUE!				
	0.046	-40.681	1.0	-40.68				
	0.045			#VALUE!	#VALUE!	#VALUE!		
Cal_2	0.190	102.159	1.0	102.16				
	0.179	91.248	1.0	91.25				
	0.185			96.70	7.716	7.98		
Cal_3	0.345	255.912	1.0	255.91				
	0.342	252.936	1.0	252.94				
	0.344			254.42	2.104	0.83		
Cal_4	0.614	522.746	1.0	522.75				
	0.615	523.738	1.0	523.74				
	0.615			523.24	0.701	0.13		
Cal_5	0.862	768.750	1.0	768.75				
	0.839	745.935	1.0	745.94				
	0.851			757.34	16.133	2.13		
Cal_6	1.145	1049.472	1.0	1049.47				
	1.121	1025.665	1.0	1025.67				
	1.133			1037.57	16.834	1.62		
Cal_7	1.667	1567.270	1.0	1567.27				
	1.645	1545.447	1.0	1545.45				
	1.656			1556.36	15.431	0.99		
Cal_8	2.035	1932.308	1.0	1932.31				
	2.103	>max	1.0	#VALUE!				
	2.069			#VALUE!	#VALUE!	#VALUE!		
48 sictrl2	0.312	223.177	10.0	2231.77			2464,66	(25µg / 2,46466) x 2
	0.373	283.686	10.0	2836.86				
	0.343			2534.32	427.863	16.88		= 20,3 µl
48 sictrl1	0.264	175.564	10.0	1755.64				
	0.260	171.596	10.0	1715.96				
	0.262			1735.80	28.057	1.62		= 28,8 µl
48 siKIT1	0.273	184.491	10.0	1844.91				
	0.262	173.580	10.0	1735.80				
	0.268			1790.36	77.156	4.31		= 27,9 µl
48 siKIT2	0.272	183.499	10.0	1834.99				
	0.250	161.676	10.0	1616.76				
	0.261			1725.88	154.311	8.94		= 29,0 µl
48 siMYB1	0.307	218.218	10.0	2182.18				
	0.282	193.419	10.0	1934.19				
	0.295			2058.18	175.354	8.52		= 24,3 µl
48 siMYB2	0.271	182.507	10.0	1825.07				
	0.272	183.499	10.0	1834.99				
	0.272			1830.03	7.014	0.38		= 27,3 µl
882 sictrl1	0.391	301.541	10.0	3015.41			1560,48	
	0.145	57.522	10.0	575.22				
	0.268			1795.32	1725.480	96.11		= 32,0 µl

x Lasketun muista mittauksista 4-5 tuloksen ka

882 sictrl2	0.440	350.147	10.0	3501.47	1675,8525			= 29,8 µl
	0.203	115.055	10.0	1150.55				
	0.322			2326.01	1662.353	71.47		
882 siKIT1	0.210	121.998	10.0	1219.98				
	0.214	125.966	10.0	1259.66				
	0.212			1239.82	28.057	2.26		= 40,3 µl
882 siKIT2	0.228	139.853	10.0	1398.53				
	0.220	131.918	10.0	1319.18				
	0.224			1358.86	56.113	4.13		= 36,8 µl
1 882 siMYB	0.296	207.306	10.0	2073.06				
	0.275	186.475	10.0	1864.75				= 25,4 µl
	0.286			1968.91	147.297	7.48		
2 882 siMYB	0.236	147.789	10.0	1477.89	1263,826			= 39,6 µl
	0.206	118.031	10.0	1180.31				
	0.221			1329.10	210.424	15.83		

- pienimmän mukaan lasketaan eli 1239,82 ng
- 10 µg per kaivo 1 osa
  10 : 1,23982 = 8,0657
- näytteet laimennetin daemmiin 1:2
  8,0657 x 2 = 16,1314
  ≈ 16,1 µl = 10 µg

RNA purity and yield

Sample of cell line 882	Yield of RNA ng/µl	Purity of RNA 260/280 nm
Validation control 1	14,7	1.92
Validation control 2	12,7	1.84
Validation control 3	10,3	2.05
Non-treated cells 1	7,30	2.04
Non-treated cells 2	7,90	1.97
Non-treated cells 3	15,7	2.05
Imatinib treated cells 1	4,40	1.53
Imatinib treated cells 2	5,60	1.72
Imatinib treated cells 3	4,70	1.62
siRNA control 1	9,00	1.95
siRNA control 2	8,00	1.99
siRNA control 3	9,20	2.08
MYB siRNA transfected cells 1	5,20	2.12
MYB siRNA transfected cells 2	2,50	1.55
MYB siRNA transfected cells 3	4,90	1.85

Sample of cell line 48	Yield of RNA ng/µl	Purity of RNA 260/280 nm
Validation control 1	16,4	1.94
Validation control 2	23,3	1.94
Validation control 3	20,7	2.05
Non-treated cells 1	18,3	2.00
Non-treated cells 2	19,3	2.01
Non-treated cells 3	13,8	1.92
Imatinib treated cells 1	4,01	2.29
Imatinib treated cells 2	5,00	1.70
Imatinib treated cells 3	4,70	1.91
siRNA control 1	19,1	1.97
siRNA control 2	25,3	1.95
siRNA control 3	17,7	2.01

MYB siRNA transfected cells 1	20,8	1.88
MYB siRNA transfected cells 2	20,3	2.08
MYB siRNA transfected cells 3	21,4	1.93

Control sample	Yield of RNA ng/µl	Purity of RNA 260/280 nm
MDA-MB-361	1893,7	2.12

96-well plate designs in qPCR

Date: 25.5.2016 Plate: 1

MYB gene Control gene *G6PD*

	1	2	3	4	5	6	7	8	9	10	11	12
A	882 IM ctrl 1	882 IM ctrl 1	882 IM ctrl 1	882 MYB siRNA 1	882 MYB siRNA 1	882 MYB siRNA 1	882 IM ctrl 1	882 IM ctrl 1	882 IM ctrl 1	882 MYB siRNA 1	882 MYB siRNA 1	882 MYB siRNA 1
B	882 IM ctrl 2	882 IM ctrl 2	882 IM ctrl 2	882 MYB siRNA 2	882 MYB siRNA 2	882 MYB siRNA 2	882 IM ctrl 2	882 IM ctrl 2	882 IM ctrl 2	882 MYB siRNA 2	882 MYB siRNA 2	882 MYB siRNA 2
C	882 IM 1	882 IM 1	882 IM 1	882 MYB siRNA 3	882 MYB siRNA 3	882 MYB siRNA 3	882 IM 1	882 IM 1	882 IM 1	882 MYB siRNA 3	882 MYB siRNA 3	882 MYB siRNA 3
D	882 IM 2	882 IM 2	882 IM 2	48 IM 1	48 IM 1	48 IM 1	882 IM 2	882 IM 2	882 IM 2	48 IM 1	48 IM 1	48 IM 1
E	882 IM 3	882 IM 3	882 IM 3	48 IM 2	48 IM 2	48 IM 2	882 IM 3	882 IM 3	882 IM 3	48 IM 2	48 IM 2	48 IM 2
F	882 siRNA ctrl 1	882 siRNA ctrl 1	882 siRNA ctrl 1	48 IM 3	48 IM 3	48 IM 3	882 siRNA ctrl 1	882 siRNA ctrl 1	882 siRNA ctrl 1	48 IM 3	48 IM 3	48 IM 3
G	882 siRNA ctrl 2	882 siRNA ctrl 2	882 siRNA ctrl 2	MDA-MB-361	MDA-MB-361	MDA-MB-361	882 siRNA ctrl 2	882 siRNA ctrl 2	882 siRNA ctrl 2	MDA-MB-361	MDA-MB-361	MDA-MB-361
H	882 siRNA ctrl 3	882 siRNA ctrl 3	882 siRNA ctrl 3	H2O	H2O	H2O	882 siRNA ctrl 3	882 siRNA ctrl 3	882 siRNA ctrl 3	H2O	H2O	H2O

Date: 25.5.2016 Plate: 2

MYB gene Control gene *G6PD*

	1	2	3	4	5	6	7	8	9	10	11	12
A	882 validation ctrl 1	882 validation ctrl 1	882 validation ctrl 1	48 IM ctrl 2	48 IM ctrl 2	48 IM ctrl 2	882 validation ctrl 1	882 validation ctrl 1	882 validation ctrl 1	48 IM ctrl 2	48 IM ctrl 2	48 IM ctrl 2
B	882 validation ctrl 2	882 validation ctrl 2	882 validation ctrl 2	48 siRNA ctrl 1	48 siRNA ctrl 1	48 siRNA ctrl 1	882 validation ctrl 2	882 validation ctrl 2	882 validation ctrl 2	48 siRNA ctrl 1	48 siRNA ctrl 1	48 siRNA ctrl 1
C	882 validation ctrl 3	882 validation ctrl 3	882 validation ctrl 3	48 siRNA ctrl 2	48 siRNA ctrl 2	48 siRNA ctrl 2	882 validation ctrl 3	882 validation ctrl 3	882 validation ctrl 3	48 siRNA ctrl 2	48 siRNA ctrl 2	48 siRNA ctrl 2
D	882 IM ctrl 3	882 IM ctrl 3	882 IM ctrl 3	48 siRNA ctrl 3	48 siRNA ctrl 3	48 siRNA ctrl 3	882 IM ctrl 3	882 IM ctrl 3	882 IM ctrl 3	48 siRNA ctrl 3	48 siRNA ctrl 3	48 siRNA ctrl 3
E	48 validation ctrl 1	48 validation ctrl 1	48 validation ctrl 1	48 MYB siRNA 1	48 MYB siRNA 1	48 MYB siRNA 1	48 validation ctrl 1	48 validation ctrl 1	48 validation ctrl 1	48 MYB siRNA 1	48 MYB siRNA 1	48 MYB siRNA 1
F	48 validation ctrl 2	48 validation ctrl 2	48 validation ctrl 2	48 MYB siRNA 2	48 MYB siRNA 2	48 MYB siRNA 2	48 validation ctrl 2	48 validation ctrl 2	48 validation ctrl 2	48 MYB siRNA 2	48 MYB siRNA 2	48 MYB siRNA 2
G	48 validation ctrl 3	48 validation ctrl 3	48 validation ctrl 3	MDA-MB-361	MDA-MB-361	MDA-MB-361	48 validation ctrl 3	48 validation ctrl 3	48 validation ctrl 3	MDA-MB-361	MDA-MB-361	MDA-MB-361
H	48 IM ctrl 1	48 IM ctrl 1	48 IM ctrl 1	H2O	H2O	H2O	48 IM ctrl 1	48 IM ctrl 1	48 IM ctrl 1	H2O	H2O	H2O

Date: 25.5.2016 Plate: 3

MYB gene Control gene *G6PD*

	1	2	3	4	5	6	7	8	9	10	11	12
A	48 MYB siRNA 3	48 MYB siRNA 3	48 MYB siRNA 3	48 MYB siRNA 3	48 MYB siRNA 3	48 MYB siRNA 3						
B	48 IM ctrl 3	48 IM ctrl 3	H2O	48 IM ctrl 3	48 IM ctrl 3	48 IM ctrl 3						
C	MDA-MB-361	MDA-MB-361	H2O	H2O	H2O							
D												
E												
F												
G												
H												

Relative expression of qPCR

48 Validation control 1	0.5282
48 Validation control 2	0.4337
48 Validation control 3	0.3256
48 Imatinib treated cells 1	0.3655
48 Imatinib treated cells 2	0.2537
48 Imatinib treated cells 3	0
48 Non-treated cells 1	0.1979
48 Non-treated cells 2	0.2190
48 siRNA control 1	0.2608
48 siRNA control 2	0.2324
48 siRNA control 3	0.1808
48 MYB siRNA transfected cells 1	0.2024
48 MYB siRNA transfected cells 2	0.1742
882 Validation control 1	0.4602
882 Validation control 2	0.9377
882 Validation control 3	0.5682
882 Imatinib treated cells 1	6.77E-2
882 Imatinib treated cells 2	0.2345
882 Imatinib treated cells 3	0.3917
882 Non-treated cells 1	0.7571
882 Non-treated cells 2	1.171
882 Non-treated cells 3	1.976
882 siRNA control 2	1.001
882 siRNA control 3	1.289
882 MYB siRNA transfected cells 1	0.2591
882 MYB siRNA transfected cells 3	0.4492
882 MYB siRNA transfected cells 2	1.082

Anniina Tervi

MYB gene expression in GIST cancer cell lines

Thesis: Project plan

Helsinki Metropolia University of Applied Sciences
Health services
Biomedical laboratory science (Bachelor degree)
Thesis
30.3.2016

Metropolia

Table of contents

1 Introduction

Meaning of cancer research is mainly to improve our own knowledge of cancer and that way find out ways to prevent the occurring of different cancers. Additionally, through research we can find ways to diagnose and treat cancer with improved ways. When we can find ways to fight back cancer, we can help to make people understand cancer better. Through open and linked global connections, we can – as a society – accomplish great actions concerning the fight against cancer. (Cancer Research UK 2009 – 2014.)

My part of cancer research comes in to the light through my thesis. Subject of my thesis is strongly linked to the research of GIST (Gastrointestinal stromal tumour) and its origins. In this particular research study, I am going to study the meaning of *MYB* gene expression in GIST cancer cell lines. Additionally, the basic function of protein production and ways to interfere this event, are examined during the research. Preliminary results of Dr. Sihto indicate that *MYB* might control transcription of the genes which are known to be associated with GIST tumorigenesis. The most common gene that causes GIST is so called *KIT* gene and about 85 – 95% of this kind of tumours arise from the mutation in the *KIT* gene. Basic purpose is to find ways to help preventing the formation of GIST cancer cells and understand more about how this particular tumour is shaped in the intestines. Furthermore, this study might help to find out more about genetics behind tumour formation, especially concerning the role of *MYB* gene.

By using cell culture, we can multiple the amount of GIST cancer cells that I am going to use in my study. The wanted knowledge of *MYB* gene expression can be detected by using western blotting. Western blotting reveals if these cells produce protein that is coded by the *MYB* gene. If these cells in fact do so, we can try reduce this protein production by blocking the messenger RNA of *MYB*. To block mRNA we can utilize *MYB* specific Small interfering RNA. Additionally, the effect of imatinib in *MYB* gene expression and blocking the mRNA is added to this study. If the amount of protein decreases, we can assume that the amount of mRNA decreases in the same proportion. When the production of abnormal *MYB* controlled protein decreases, we might be able to see the effects of it in cell apoptosis or in cell proliferation in GIST cancer cells.

This study is conducted to the research group of Heikki Joensuu, University of Helsinki. Purpose of this study for the research team is to gain more knowledge of *MYB* gene-function. They already have supporting evidence concerning my thesis and this evidence is the basic idea behind thesis. Already have been noticed in minor amounts that GIST cells produce the protein that is controlled by *MYB* gene. Protein production was discovered through western blotting. Also early evidence has shown that the amount of mRNA decreases when e.g. siRNA is added to the cell culture. To reinforce this idea and to help deepen this evidence, I am going to repeat these analysis with more material during three weeks of practical laboratory analysis. My results will hopefully improve research team's purpose of resolving the origins of GIST.

My thesis consist of firstly this project plan, laboratory tests, analysing the results and then reporting them. In the final report I am going to demonstrate the materials, methods and analysing process accurately and finally discuss the matter and hopefully have a conclusion to my thesis. Information of this thesis is used to study more about *MYB* gene expression and in the further research of GIST. My priority is to figure out impact of *MYB* gene expression on GIST. If it indeed does have a considerable effect, we must ask ourselves how, why and under what conditions it effects GIST cells.

Personally, I am aiming to increase my knowledge of transcription factors, interference of cancer cells, protein formation and also increase knowledge of siRNAs and Imatinib medication. On the side, the methods like cell culture and western blotting will come hopefully clearer to me. Generally, working in a research team and learning new ways of working, are my goals during the practical part of my study. I am also aiming to bring forth profitable information to help successfully GIST research. This thesis might work as a good base for the future research of *MYB* gene expression's meaning in the formation of GIST.

2 Background

The most recognised ability of cancer cells is their way of sustaining chronical proliferation. Normally tissues control their cell production and are able to release growth-promoting signals that can guide the cell's growth and division cycle. This release and control help to maintain homeostasis of the cell number and normal structure and function

of the cell. Cancer cells disturb these signals and this way determinate their own destinies. This chronic proliferation can be sustained in multiple ways: they can produce growth factor signals, they can send signals to stimulate normal tissues, and they canincrease the levels of receptor proteins (in the cancer cell surface) and become hyperresponsive towards growth factors. (Hanahan – Weinberg 2011.)

Normal cells also use negative-feedback to control their homeostatic balance. Damage in negative-feedback systems are able to enhance proliferative signalling e.g. in cancer cells. Increasing expression of oncogenes and their protein production signals, on the other hand, can effect directly on elevated cancer cell proliferation and in that way to tumour growth. Additionally, cancer cells have to come up with programs that negatively monitor cell proliferation. These actions are partially dependant on the cells disappeared action of tumour suppressor genes. Above all cancer cells need nutrients and oxygen and a way to lose metabolic wastes that angiogenesis provides in the form of tumour-associated neovasculature. (Hanahan – Weinberg 2011.)

2.1 GIST

GIST is an abbreviation for gastrointestinal stromal tumour. GISTs are gastrointestinal mesenchymal tumours that embody CD117 (*KIT*) proto-oncogene. GIST tumours are just 1 to 3 % of all of the GI tumours, but it is the most common soft tissue sarcoma of the gastrointestines. It is believed that GIST tumours originate from precursor cells of interstitial cells of Cajal, which are in control of spontaneous abdominal peristaltic movements. Usually (50 – 60%), the treatment of GIST, includes the removal of the tumour. Additionally, imatinib can be used in metastatic GISTs or in GISTs that have a high risk of regeneration. (Lääkärin käsikirja 2014; Sihto 2016.)

2.1.1 GIST diagnosis

Generally GISTs are caused by active mutations in the *KIT* or *PDGFRA* genes. These tumours are normally found in stomach (60 %), small intestine, jejunum and ileum (30 %), duodenum (5 %), rectum (2–3 %) and from the colon (1–2 %). GISTs consist of a group of neoplasms with various morphology, behaviour and genetic features. On histopathologic view, GISTs are epithelioid-, spindle-, or mixed-cell tumours. These tumours

can be categorised as malignant, borderline or benign depending on its size, invasion to nearby tissues and mitotic index. (Poveda et al. 2013.)

The diagnosis of these tumours mostly relies on its histopathological features and on the immunohistochemical phenotype. Anoctamin-1 (ANO1/DOG1) is known marker for GISTs and is used in differential diagnosis of GIST. Moreover, determination of *KIT* and *PDGFRA* mutations, are strongly connected to GIST diagnostics. These mutations are helpful to confirm diagnose in uncertain cases. The mutations can also help determinate the response of imatinib in tumours. (Gong – Li – Zhao – Zhao – Zhang 2009; Sihto 2016.)

2.1.2 Previous knowledge of GIST

Ostrowski et al. studied molecular characteristics of type three tyrosine kinase receptor that is connected with known GIST mutations in KIT and PDGFRA. Both mutations are thought to promote early oncogenic events in similar pathways. Purified RNA from the GISTs and sequencing of mRNA was used to clarify these KIT and PDGFRA mutations. Most of the tumours possessed either or both of these mutations. Gene expression altered between two groups of GISTs at some levels e.g. considering angiogenesis. Their study found new molecular elements that might be involved in receptor-dependent GIST evolution and also backed up previously known information about GIST receptors. These molecular elements might be good therapeutic targets and markers of KIT mutation status. (Ostrowski et al. 2009.)

Although, GISTs are usually localised in the GI tract, Gong et al. in year 2009 released an article about a GIST tumour found from sacrum in China. This particular tumour showed positive for both CD117 and CD34 proteins. Additionally, tumour showed positive morphological aspects for being GIST tumour and tumour was found positive in PCR of the mutation in c-KIT gene in exon 11. These facts strengthened the realization of this tumour being in fact GIST. In this study also brought to surface the clonal origins of GIST. GIST is (as shown before) considered to be a mesenchymal tumour and in this study GIST was proven to be monoclonal. Furthermore, 15 separate tumour nodules expressed loss of similar X chromosomal inactivation mosaicism. (Gong et al. 2009.)

2.2 MYB gene

MYB is a proto-oncogene that is located on chromosome 6q in human genome. Its main function is to encode a 72 kDa nuclear binding transcription factor. Transcription factor in question is identified to be a sequence-specific DNA-binding transcriptional regulator protein in human cells. In this factor there are three main functional areas; amino terminus as a DNA binding domain, mid portion as a transcriptional trans-activating domain and also carboxyl terminus as a negative regulator. *MYB* is especially found in hematopoietic cells and *MYB* expression is particularly high in immature hematopoietic cells. Recently *MYB* has been found also from non-hematopoietic cells and from cancer cells involving lung, breast, colon carcinoma and melanoma. (Kim et al. 2008.)

2.2.1 Function of *MYB* gene

The family of *MYB* genes consist of three different types of *MYB*, which are named A, B and c-myb. All of these genes encode proteins that are involved in transcription. In my study I focus on the gene c-myb and that particular gene is called in this thesis the *MYB* gene. The transactivation of different *MYBs* varies highly on different cell types and in promoter contexts. This variability between different *MYBs* suggests that there are also dependence with *MYBs* and other cell type specific co-factors e.g. interaction with other transcription factors. Gene expression of these genes is noticed to be cell cycle-regulated. If this expression is inhibited with anti-sense oligonucleotides, it may effect on cell-cycle progression, cell differentiation and cell division. (Oh – Reddy 1999.)

V-myb is a transforming homolog oncogene of *MYB* that is connected to avian myeloblastosis virus (AMV) and to avian leukaemia virus E26. These viruses are strongly connected to leukaemias. The so called v-myb genes are able to transform haematopoietic cells in tissue cultures and causes leukaemias in animals. Because the MYB protein is known to be a DNA-binding transcription factor, its oncogenic activity might be linked to its capacity to monitor specific target genes that impact cell reproduction or tumorigenesis. (Kim et al. 2008; Quintana et al. 2011.)

2.2.2 Role of *MYB* in cancer

In research study of West et al. *MYB* gene was examined in adenoid cystic carcinoma (AdCC). It was discovered that translocation t(6;9) (q22-23;p23-24) was connected to multiple cases of AdCC. Moreover, the fusion between transcription encoding *MYB* gene and NFIB transcription factor in 6/11 occasions of AdCC was discovered. At that time only a potential pathogenic translocation suggested it to be a useful diagnostic advantage for differentiation of AdCC. In the study of West et al. *MYB* translocation was searched by increased levels of *MYB* expression. *MYB* translocation was discovered from one-half of the AdCC cases (49 %), but from the other salivary gland tumours there were no *MYB* translocation to be found. All in all, 65 % AdCCs have an unusual *MYB* FISH pattern, which indicates *MYB* translocation. (West et al. 2011.)

Hudson and Collins studied furthermore the linkage between *MYB* gene and adenoid cystic carcinoma. Their main focus was to identify more diagnostic means to identify salivary gland basaloid neoplasms. CD117 with morphological aspects from fine needle biopsy are the most common ways to identify AdCC. Also AdCC tumours have a DNA abnormalities t(6;9) (q22-23;p23-24) translocation, which can be used as a diagnostic marker of this tumour. This particular translocations contains *MYB* gene that encodes transcription factors and is therefore specific for AdCC in salivary gland neoplasms. (Hudson – Collins 2014.)

In Hudson – Collins study there were ten samples that were diagnosed as AdCC by using FNA (Fine needle aspiration) samples. Of these FNA samples, 50 % (5 cases) showed *MYB* gene abnormalities. These FNA samples were also compared with the most common diagnose method ergo histopathological analyse. It was furthermore pointed out that t(6;9) translocation is discovered also in adenoid cystic carcinoma of the breast as well as in the salivary gland. This translocation in question might make fusions to new gene partners. This fusion might lead to loss of control of the *MYB* gene expression, which is likely to effect to the neoplastic process in AdCC. (Hudson – Collins 2014.)

Quintana et al. studied the role of *MYB* gene in breast cancer and *MYB* connection to estrogen levels and estrogen response in breast cancer cells. Their idea was that expression of *MYB* gene is associated with the estrogen receptors (ERs) in breast tumours. The *MYB* gene reacts to the activation of ERs in certain known breast cancer cell lines

like MCF-7. MYB transcription factor (shown in this study) is thought to be connected in the regulation of many different gene expressions that are important in breast cancer evolution and progression. Thus, when *MYB* gene is activated and starts to produce transcription factor, other genes react to this transcription factor and start e.g. producing breast cells in an uncontrolled way. Linkage to ERs in breast cancer may also indicate that MYB transcription factor is regulated mainly by different protein to protein interactions. These Interactions may help to guide MYB transcription factor to different targets in multiple tissues. (Quintana et al. 2011.)

Generally, there is only little known about *MYB* in tumorigenesis in human cells. More studies and advanced techniques may be required to resolve the mysteries of *MYB*. More results may help to understand which kind of signals and regulators are important in the role of controlling the activity of MYB transcription factor. However, it is not yet clear does the MYB transcription factor regulate genes that are involved in tumour growth. (Quintana et al. 2011.)

2.3 Imatinib and siRNA

Imatinib is a drug that is used in drug therapy of chronic myeloid leukaemia (CML) – that is Philadelphia-chromosome positive –, GISTs and acute lymphoblastic leukaemia etc. Imatinib is a kinase inhibitor that works by blocking the function of kinases with abnormal enzymatic activity. Target proteins for imatinib are i.a. oncoprotein BCR-ABL (connected with CML), KIT and PDGFR. These targets can be in charge of giving cancer cells signals to multiply without control. Imatinib helps preventing the uncontrolled way of growth in cancer cells. (Imatinib (Glivek) 2015; Sihto 2016; Lee – Wang 2009.)

SiRNA is a small interfering RNA which job is to interfere expression of protein coding genes. SiRNA is a synthetic duplex RNA designed to attach identical corresponding mRNA in sequence to undermine its function. When attached, siRNAs can block the protein production. SiRNAs can also knockdown gene expression by causing promoter genes methylation and chromatin condensation. Their ability to induce gene knockdown is limited to cells that are receptive to transfection of synthetic oligonucleotides. (Ross – Carlson – Brock 2007; Dharmacon 2016.)

2.4 Information retrieval

I have been searching information for this thesis throughout the process. Articles and facts will be increased during the practical and reporting stage of this process. Mainly my search is restricted to different data bases on the internet. Helsinki Metropolia University of Applied sciences has open access to the data bases that I have used in my information retrieval. If possible, I will be using data bases of University of Helsinki during my practical part in research group of Heikki Joensuu. Some retrieval will be executed in different libraries e.g. in the library of Metropolia and in the library of University of Helsinki.

3 Materials and methods

Cell lines for my study are provided by Jonathan Fletcher MD from United States of America. Cell lines for imatinib-sensitive GIST (GIST882) and GIST (GIST48) are assured by sequencing GISTs using their already known mutations spots. GIST cell lines are separated from human GIST cancer cells. (Bauer – Duensing – Demetri – Fletcher 2007; Sihto 2016.)

Received results will be analysed by using SPSS statistical program and also by using Microsoft Excell program.

3.1 Cell culture

In cell culture, cells are grown in an optimal artificial environment. Cells are removed from an animal or humans and they can be removed from tissues directly. Before the cultivation, cells can be manipulated by enzymatic or mechanical means or they can be separated from a cell line that has already been established. The condition to grow cells vary depending on the cell type in use. The artificial environment to grow cells though contains primarily the following aspects:

- A substrate that includes essential nutrients (e.g. amino acids and carbohydrates)
- Growth factors
- Gases (oxygen and carbon dioxide)
- Hormones

- Regulated environment (pH, osmotic pressure, temperature)

(Thermo Fisher Scientific 2016.)

To culture cells in laboratory environment, the laboratory must have certain aspects. Laboratory is recommended to be dust free and have no through traffic that can have a harmful effect to the cells. Laminar flow hoods allow these aspects to be fulfilled. Laminar flow hoods provide also an aseptic area for working with tissues. The laboratory should also be separated from the preparation place, wash-up and sterilization places, while still being close to each other. (Freshney 2010:25, 37.)

An artificial substrate is a base where cultured cells can grow as monolayers or as a suspension. That is why the substrate must allow cell adhesions, which allows cells to grow and spread throughout the surface. The most commonly used substrate materials are disposable plastic and glass. These materials can be pre-treated with e.g. nutrients that improve cell attachment and growth. (Freshney 2010: 89–90.)

Growth factors in serum stimulate cell proliferation, especially those factors that are platelet-derived (PDGF – platelet-derived growth factor). PDGF is known to stimulate growth particularly in glia cells and also in fibroblasts. Other growth factors such as fibroblast growth factors and epidermal growth factors are to be considered in cell cultures. Many of these factors are available for buying as recombinant proteins. Hormones that have a mitogenic feature, like insulin, growth hormone and hydrocortisone, are used in cell cultures. Insulin improves the up taking of glucose and amino acids. Hydrocortisone for its part, can promote cell to cell attachments. (Freshney 2010: 111.)

As for the controlled and stable environment of the cell culture, cell type specification define the used physical aspects. Commonly most cells are accustomed to grow well at pH 7.4. Carbon dioxide helps lowering the pH by dissolving into bicarbonates. Additionally, oxygen is required to maintain the cell respiration. Although, it is important to stable the oxygen level to the stage where it helps respiration but does not release toxins to the culture. The variation in osmolality is widely tolerated among different cells, but recommended osmolality in culture is between 260 mOsm/kg and 320 mOsm/kg. As for temperature, it is recommended to resemble the original source of the cells (growing human cells temperature is optimal in 37 Celsius). (Freshney 2010: 99–106.)

In my study cell culture will be based on aseptic work methods. GIST cells are grown in suitable environment (RPMI base). In to the medium of cell culture, heat inactivated FBS and suitable antibiotics are added. Cells are to be maintained by dividing them and by changing the base on regular basis. To this cell growth environment, imatinib can be added and incubated few days to let it effect on the cells of the culture. Small interfering RNAs are transfected to the cells by using Lipofectamine 2000 reagent. After the transfection, cells are left also for few days before any further tests can be performed that the gene or protein expression has time to fade. (Sihto 2016.)

3.2 Western blotting

Western blotting is a technique that is used to separate wanted proteins based on a mass of proteins. Technique is based on transferring proteins from the separating gel onto a supporting matrix. This blotting style allows for fast staining and for destaining protocols of the separation of proteins. In western blotting, protein samples are separated in a gel through gel electrophoresis and after that electroblotted onto a support matrix. Once this is done, proteins can be labelled with anything that is able to selectively bind into peptides to identify the presence or absence of specific spots. This detection of proteins can be reached via an enzyme fit for use with a changing ways of colorimetric, chemiluminescent or fluorescent substrates. (Walker – Rapley 2005: 43.)

After the gel electrophoresis, protein transfer from the gel is accomplished by using an electric potential through the gel and the membrane (support matrix see above) – where proteins are transferred to – which are in contact with each other. The applied membrane can be nitrocellulose, nylon or polyvinylidene difluoride (PVDF) base. This membrane provides much more stable environment than the gel, making later manipulations considerably easier. Electroblotting is the most common western blotting technique, but also techniques such as capillary blotting can be used. (Walker – Rapley 2005: 43–44.)

Following transfer to the membrane, finding of target proteins is commonly accomplished by using specific antibodies (protein labelling see above). The implantation of these specific antibodies to their target proteins can be performed by using methods that are based on indirect or direct immunoassays. Immunoassay methods include the using of multiple

labels that are conjugated to an antibody. In direct method, the signal of these labels is noticed directly when the antibody attaches itself to the protein that is looked for. When in indirect immunoassay methods, the signal can be achieved by using enzymes that can act on particular substrates. (Walker – Rapley 2005: 46.)

In my study I am going to use SDS-PAGE gel electrophoresis and the used gels are directly bought from the Citylab. Gels are made by the Biorad Company. The used secondary antibody is labelled with HRP (horseradish peroxidase) and the chemiluminescent reaction on membrane on each protein is accomplished by exploiting enzymatic reactions. The magnitude of the reaction is directly correlated to the amount of protein in the sample. This chemiluminescent reaction is identified by using x-ray film. (SIhto 2016.)

3.3 Permissions and reliability

For my study I do not require any ethical permissions etc., because materials are not from patients, no animal testing is included nor genetically modified organisms are included in my study. Reliability of my study depends on my materials, manners of my working and also on my references. These parts are processed in my reports discussion part at the final stage of my thesis.

3.4 Schedule

Schedule, concerning my working, is divided into four major parts. First there was an introduction to this subject, now I am planning my project, at May I am going to proceed the practical part of my study and at autumn will be the final reporting and writing of my thesis. I have scheduled my meetings with my instructors and made a quite accurate timetable to find information and to write my report. My schedule is also shown in Table 1.

Table 1. Schedule

2016	Meetings	Information retrieval	Writing	Execution	Due days	Seminar
Introduction to the subject	Harri: 4.2 Hannele: 16.2	7.2 – 19.2	16.2 – 25.2		25.2	1.3.
Project plan	Hannele: 22.3	week 10	10.3 – 29.3		31.3	5.4 – 6.4
Execution		April – May		May weeks 19 – 21		
Reporting and Final work		June – August	June – October		8.11 24.11	15.11 – 16.11

4 Publication and communication

This thesis will be published November 2016 at Vanha Viertotie campus of Helsinki Metropolia University of Applied Sciences. Thesis will be also available for the research group of Heikki Joensuu for future research on this subject. Online this thesis will be published to Theseus database. If recommended or needed, this thesis could be published also somewhere else. Throughout this project I will be mentored and instructed by Harri Sihto from University of Helsinki, Laboratory of Molecular Oncology and by Hannele Pihlaja instructor of my thesis in Helsinki Metropolia University of Applied Sciences. Communication will be performed through email correspond and with regular meetings with both of my instructors.

5 Discussion

There are still some unclear subjects and open questions to my thesis. These subjects and questions are for the time being presented in this chapter.

Questions and unclear subjects of my thesis:

- Will there be enough time to finish this project?

- Will my goals and abilities meet satisfactorily?
- Will my information base be enough to meet the required standards?
- Will there be enough time to make maybe more research in my thesis?
- The use of statistical programs to decode my results
- Succeeding in my practical works
    - How will imatinib and SiRNA react to GIST cells?
    - Succeeding in cell culture and western blotting
- Further studies concerning GIST and MYB linkage

5.1 Risk analysis

To analyse possible risks linked to my thesis, I have made a SWOT-analysis to knowledge these risks. My main strengths, concerning this project, are the motivation that drives me to do this thesis satisfyingly to myself, eagerness to learn new things and being interested on the subject of this thesis. Moreover, well planned schedule, wide amount of references and organizing will help me throughout this project. Main weaknesses might be the limited amount of time that I have, stress, totally new subject to work with and the amount of work I have and want to put to this project.

On the other hand, I see this project as an opportunity work in research group, which is a quite different and looked for environment to work in. Additionally – to learn new methods, to learn about cancer and gene function and also to work with new people – are great opportunities to me concerning my future as biomedical laboratory scientist. With opportunities there comes also threats that need to be seen. There is a threat that I will not be able to succeed in my goals in the limited amount of time given. Furthermore, possible disagreements and difficulties in finding consensus with people involved, is a threat that needs to be prevented and seen beforehand. My SWOT-analysis is also shown in a table form in Table 2.

Table 2. SWOT-analysis

Strengths - motivation - eagerness to learn - interesting subject - well planned schedule - wide amount of references - organizing skills	Weaknesses - limited amount of time - stress - unknown subject before - amount of work
Opportunities - working in a research group - learning new methods - learning about cancer and genes - working with new people	Threats - not succeeding my goals - limited amount of time - possible disagreements and not finding consensus with instructors

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