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Long non-coding RNA and breast cancer

An experimental research on the effect of pre-treatment with Gefitinb and Trastuzumab before stimulation with EGF on expression of NEAT1 in cells from human adenocarcinoma

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Prefase

As medical students at UiT – the Arctic University of Norway, we have to write an assignment the tenth semester, where we get the opportunity to immerse ourselves on a subject or in an area where we have particular interests. I have chosen to work with medical biology and experimental research. In that regard I was so lucky to be able to work with *The RNA and molecular pathology (RAMP) research group, Department of Medical Biology, Faculty of Health Siences, UiT – the Arctic University of Norway,* under the leadership of associate professor Maria Perander.

As always with experimental research, it is impossible to say in advance what results you get, and which once that can be used to draw any conclusions. Far from everything goes according to the plan with an inexperienced student in the lab, but there are still plenty of points to be raised in the discussion section, and I believe that I can draw some conclusions from the results I've managed to produce as well.

Thanks to everyone at the RAMP-group for making it possible for me to do this assignment, especially Maria Perander who gave me the opportunity and supervised me through this educational experience. I'm forever grateful to Mohammad Seyed Lellahi, Erik Knutsen, and last but not least Annica Hedberg, for all your help, support and patience in the laboratory during these months.

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Epitome

Breast cancer represents 22 % of all cancer cases amongst the female population in Norway, which makes it by far the most common form of cancer in this group (1). It is a heterogeneous disease (2), and to guide use of adjuvant treatment, breast cancer patients are categorized into different subgroups based on biomarkers, which in turn enables specific target treatment options. Long non-coding RNAs (lncRNA) are previously unexplored transcripts from the mammalian genome, but are currently receiving increased attention, as it potentially can give rise to novel biomarkers and new targets for medical treatment amongst cancer patients in the future. Nuclear enriched abundant transcript 1 (NEAT1) is an lncRNA that proves to be involved in tumorgenesis, as induction of it leads to accelerated cellular proliferation, improved clonogenic survival and reduced apoptosis (3). High tumor NEAT1 expression also correlates with pore survival in patients with breast cancer (3).

I have worked with two different cell lines, HeLa cells which are human cervical epithelial cells, and SK-BR-3 cells from human adenocarcinoma in breasts. I have pre-treated them with two different drugs, *Gefitinib* and *Trastuzumab*, and stimulated them with epidermal growth factor (EGF). By RNA isolation and RT-qPCR I've detected the expression of NEAT1_2 relative to GADPH, which is a constitutively expressed housekeeping ncRNA. I've also done Western Blot to detect ERK- and phosphorylated ERK proteins, to verify that EGF succeeded in stimulating the cells though activation of the Ras-MAPK signalling pathway.

HeLa cells both untreated and pre-treated with Gefitinib, and stimulated with EGF, show a transient induction in NEAT1_2 expression. SK-BR-3 cells also show a transient induction without Gefitinib treatment, whereas the cells exposed to drug show no increase in expression of NEAT1_2. Trastuzumab does not succeed in inhibiting activation of ERK to phosphorylated ERK in SK-BR-3 cells, and these cells show a light induction of NEAT1_2 expression after stimulation with EGF.

As my experiments are done as a pilot study and are only performed once, it is impossible to draw any conclusions with statistical significance from my work. Even though the results would have to be reproduced in order to conclude, they can still be used to give rise to new working theses on how the mysterious lncRNA NEAT1_2 play a role in breast cancer.

Introduction

Breast cancer

Breast cancer represents 22 % of all cancer cases amongst the female population in Norway, which makes it by far the most common form of cancer in this group (1). Today's numbers suggests that every 10th to 11th woman would be expected to get the disease (4). In 2014, 3324 Norwegian women were diagnosed with breast cancer, which is a marked increase in the incidence from 1235 cases in 1970 (5). The total cause for this increase is probably multifactorial, and it is important to keep in mind that the mammography-screening program that has been offered to all Norwegian women between 50 and 69 years from 1997 undoubtedly has increased the rate of disease detection.

In the course of 2014 there were 663 Norwegian women who died from breast cancer (5). The 5-year survival without signs of relapse is more than 80 % for all stages, but the prognosis is largely dependent on stadium (4). For comparison, the five-year relative survival for 2008-2012, where the disease is confined to the breast (stage 1), is 99.1 %, versus 26.6 % if there are distant metastases at the time of diagnosis (stage 5). These numbers suggests that improvement of early disease detection, and targeted therapy strategies to prevent proliferation and metastasis as a part of cancer treatment, could potentially have a tremendous effect on breast cancer survival in the future.

Breast cancer is a heterogeneous disease (2). To guide the use of adjuvant treatment, breast cancer patients are categorized into different subgroups based on biomarkers, which in turn enables specific target treatment options. The estrogen receptor (ER) and the progesterone receptor (PgR) are hormone receptors (HR), which in addition to human epidermal growth factor receptor 2 (HER2) and the nuclear protein Ki67 are used as routine biomarkers for clinical purposes today (6). They divide breast carcinomas into four major intrinsic subtypes: luminal A, luminal B, HER2-enriched and basal like. The luminal types are HR-positive (HR+), and the low-risk A type shows high ER-regulated gene expression, whereas the high-risk luminal B type has low ER-regulated gene expression (2). HER2-enriched carcinomas show an overexpression of HER2, and the basal like subtype is triple negative (HR- and HER2-).

Adjuvant drug treatment of localized breast cancer depends on which of these subgroups the patient belongs to in addition to age. There exist a number of endocrine treatment principles for hormone sensitive breast cancers; including *Tamoxifen,* which were the first one of it's kind. There are also different chemotherapy regiments, and in recent years more targeted treatment, like antibodies and small molecular drugs, have become available. At current time we don't have any medication for curative purposes for metastatic breast cancer (4).

Non coding RNAs

Development of new and more advanced techniques to perform whole genome transcriptome sequencing, have in a way challenged what the molecular biology calls *the central dogma* (7). That is, that the flow of genetic information in a cell is exerted by transcription form DNA to messenger RNA (mRNA), followed by translation of mRNA into proteins (8). Transcriptome studies show that the vast majority of transcribed DNA in eukaryote cells are non-coding RNA (ncRNA) (9), and in combination with the fact that only approximately 1,5 % of the human genome encodes proteins (10, 11), these non-coding elements cannot simply be dismissed as transcriptional noise. We are currently on the steps of understanding the complexity of how an organism is regulated not only by the protein coding parts of the genetic material, but by the non-coding transcripts as well. There are still exiting and unexplored parts of the mysterious and complex eukaryote cell, and more research on this field can potentially give rise to novel biomarkers and new targets for medical treatment of cancer patients in the future.

ncRNA is divided by length into short ncRNA, less than 200 nucleotides (nt), and long non-coding RNA (lncRNA) longer than 200nt. Short ncRNA, such as microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs) have emerged as a big class of epigenetic key factors over the past two decades (12), as they regulate gene expression at both transcriptional and posttranscriptional levels (11, 13). The non-coding transcriptome also includes housekeeping ncRNAs that is essential for the cells fundamental biology, such as transfer RNA (tRNA) and ribosomal RNA (rRNA), and are usually expressed constitutively (10, 14).

Long non coding RNAs

LncRNA are previously unexplored transcripts from the mammalian genome, but are currently receiving increased attention. The majority is transcribed from RNA polymerase II promoters, and they are processed by 5' capping, polyadenylation and splicing (15). They are often classified according to their location relative to protein-coding genes nearby (16). If transcribed from loci *overlapping with protein coding genes*, they can be both sense and antisense, depending on the direction of transcription relative to the protein-coding gene. Others arise form *intronic* regions inside of a protein-coding gene, transcribed in either direction. There are *intergenic* lncRNAs that are encoded by independent loci in the genome, and finally *divergent* lncRNA that are transcribed in the opposite direction form the promoter of a protein-coding gene. (*Figure 1*)

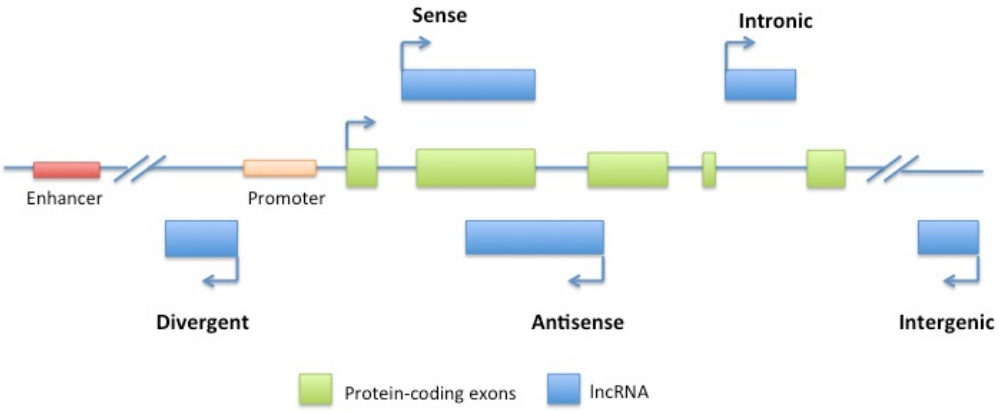

Figure 1 (modified from Maria Perander): Genomic locations of lncRNAs showing how they can be classified based on where they originate form in the genome

Defining lncRNAs based on size, the lack of potential to encode proteins and where they originate from in the genome is not a very satisfactory classification. Another, slightly more useful way of dividing lncRNAs into different groups, is by separating them based on how they function in cells. Our current imperfect understanding of these functionally heterogeneous molecules still requires a broad categorization.

Some lncRNAs are "guides" that recruit proteins and localize them to specific DNA sequences or other RNA molecules, either in *cis* (on neighbouring genes) or *trans* (genes located on different chromosomes). The regulatory proteins brought on by lncRNA-guides can be both repressive- and activating modulators, or they can be transcription factors. LncRNAs might also function as "enhancers", which stimulate the transcription of certain genes by binding to enhancer elements. Others act as "decoys" that binds and titrate away a

protein targets, almost like a sink for different regulatory factors, such as transcription factors and chromatin modifiers. LncRNAs can allosterically modify protein activity. Finally, some serve as central platforms where relevant components can be brought together on so-called "scaffolds", by possessing different domains and bind distinct effector molecules at the same time. This might have repressive or activating effects on transcription (11) (*Figure 2*).

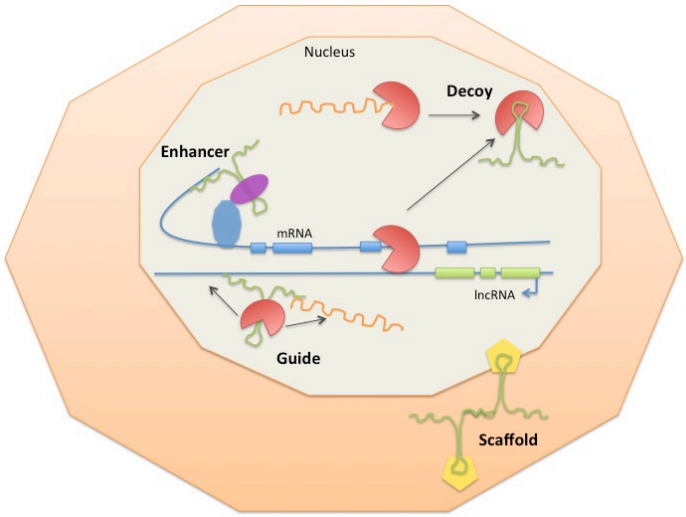

Figure 2 (modified from Maria Perander): Functional traits of lncRNAs, illustrating how they can work as enhancers, decoys, guides and schaffolds to regulate and affect gene expression within a cell nucleus.

In contrast to the short ncRNAs which are highly conserved and silence target genes through specific base paring, the lncRNAs are poorly conserved and exert their function by diverse mechanisms (11). They mediate interaction with DNA elements, other RNA molecules, or proteins either by complementary base pairing or by adapting specific structures. Recent publications have described important roles for lncRNA in regulation of transcription, splicing, nuclear and cytoplasmic trafficking, translation, chromatin organization, and as decoys of proteins and miRNAs (9, 11, 17), thus affecting important processes such as cell cycle, cell differentiation, survival, migration and metabolism (13). Increasing studies also indicate their important roles in a wide range of different biological processes, such as stress response, development and embryonic stem cell pluripotency (13).

Even though lncRNAs show different functions and mechanisms of action, the vast majority regulates expression of specific genes at different levels (16). Several studies reveal that the majority show tissue specific expression patterns and are expressed at specific points during embryonic development (13). A number of lncRNAs also display evolutional conservation and strict biological regulation, as they show spatial- and temporal-specific patterns of expression (7, 9). Giving this, it is not surprising that abnormal expression of lncRNAs is associated with numerous diseases, particularly cancer (9, 13, 18).

Cancer and lncRNAs

Cancer arises due to disruptions in cellular homeostasis, and is a multistep, multifactorial and extremely complex disease. Genes involved in cellular transformation are classified either as oncogenes that positively regulates cell cycle, or tumor suppressor genes that has a negative effect on cell cycle and survival. As for coding RNAs, genetic studies show that mutations in lncRNAs primary sequences may affect their function (7). Accumulating evidence suggests that cancer pathogenesis is associated with altered expression of lncRNAs. Normal tissue and cancer tissue show different expression of lncRNAs, which indicate that they might have a key role in cancer development (13). Both small- and large scale mutations, and dysregulations of the lncRNAs can provide altered expression, which in turn interfere with specific oncogenic- or tumor suppressor pathways in the cell (16).

It has been found that different lncRNAs can be upregulated in certain cancers, they can correlate with metastasis or poor survival, or they can indicate increased risk of recurrence (13). This knowledge provides a potential use of lncRNAs as novel biomarkers of malignancy in clinical practice. It's always important to improve methods to ensure early diagnosis and prognosis prediction for cancer patients, and further investigation of the roles and mechanisms of these peculiar elements can also provide new lncRNA-based, personalized, treatment strategies.

NEAT1 and paraspeckles

Nuclear enriched abundant transcript 1 (NEAT1) is a lncRNA transcribed by RNA polymerase II from the genetic locus *familial tumor syndrome multiple endocrine neoplasia (MEN) type I,* on human chromosome 11 (19). There are two isoforms produced from the same promoter, NEAT1_1 that consists of 3700 nt, and the larger NEAT1_2 at the size of 23 000 nt (20). Both isoforms are single exon transcripts, thus lacking introns, and are processed at the 3' end resulting in canonically polyadenylated NEAT1_1 and non-canonically processed NEAT1_2 (19). Neither are transported to the cytoplasm, but rather retained in the nucleus of the cell (19), where they associate with proteins to form structures called *paraspeckles* (21).

The nucleus in complex eukaryote organisms is highly organized, and nuclear organization is linked to genome maintenance and control of gene expression (21). It's not surprising that disruption of nuclear organization is correlated with disease stages, as it influences growth, development and cellular proliferation (21). In addition to individual chromosomes in the nucleus, there are specific proteins and nucleic acids enriched in subnuclear structures, which are membrane-less compositions called *nuclear bodies*. Paraspeckles are ribonucleoprotein bodies found in the interchromatin space of mammalian nuclei. Numerous lncRNAs are found to localize to specific nuclear bodies, amongst them are NEAT1, which forms an essential structural component in paraspeckles (20, 21). *Drosophila melanogaster behavior human splicing* (DBHS) proteins are paraspeckle proteins that rapidly target NEAT1 once it is transcribed. The finished paraspeckle likely consists of NEAT1 RNA-DBHS protein complexes that form a structural scaffold in the nucleoplasm (21). (*Figure 3*)

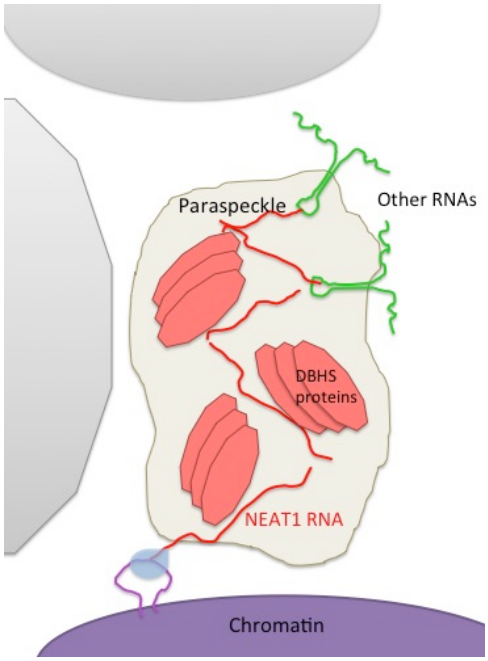

Figure 3 (modified from "Paraspeckles: nuclear bodies built on long noncoding RNA" The Journal of Cell Biology): an schematically model of a paraspeckle within the interchromatine space

Although considerable effort is put into understanding the composition, formation and molecular organisation of the paraspeckle, the biological function and the role of NEAT1 remain to be fully explored. Paraspeckles are thought to regulate expression of certain genes through nuclear retention of specific RNAs(21). Based on current knowledge, one can say that NEAT1 works as both a scaffold and a decoy within the paraspeckle. Paraspeckles are currently considered to be a unique lncRNA-directed nuclear body that is potentially involved in stress response (20). In 2009, Chen and Carmichael found that mRNA that was retained in the nucleus, and normally released due to different stress responses, relocated to the cytoplasm after NEAT1 knockdown and dissolution of paraspeckles (22). Cells are shown to be more sensitive to proteasome inhibitor-mediated cell death in the absence of paraspeckles, which further suggests a pro-survival role of paraspeckles under different stresses (20).

NEAT1 is already shown to be involved in tumorgenesis, including leukemia (23) and prostate cancer (24). It is also induced upon immune responses due to viral infections, such as HIV (21). A few years back Choudhry and colleagues(3) found that tumor hypoxia induces paraspeckle formation through activation of NEAT1, leading to cancer cell survival through accelerated cellular proliferation, improved oncogenic survival and reduced apoptosis, in several breast cancer cell lines and solid tumors. Activation of hypoxia pathways is a common feature in many cancers and frequently correlates with poor survival (3). Ke and colleagues(25) also found that downregulation of NEAT1 in breast cancer cells inhibits cell growth and induces cell apoptosis. They believe that their research indicates that NEAT1 is required for survival of breast cancer cells.

NEAT1 knockout mice that lack paraspeckles are viable and fertile but stochastically fail to become pregnant despite normal ovulation (26). Standaert and colleagues(27) recently published results showing that paraspeckles are assembled in luminal epithelial cells during mammary gland development. Furthermore they found aberrant mammary gland morphogenesis and lactation defects after genetic ablation of NEAT1. Interestingly, they provided evidence that the lactation defect is caused by a decreased ability of NEAT1-mutant cells to sustain high rates of proliferation during lobular-alveolar development.

The Ras-MAPK signalling pathway

Epidermal growth factor (EGF) binds and activates a type of membrane receptor called tyrosine-kinase-receptors (TKR) (28). After activation, the TKR recruit intracellular signalling proteins, which in turn binds and activates other proteins that pass the message along. The Ras protein is bound by a lipid tail to the cytoplasmic side of the plasma membrane, and is a key player in adaption-assembled signalling complexes (29). It cycles between an active state when GTP is bound, and an inactive state when bound to GDP (30). While active, Ras promotes the activation of a phosphorylation cascade called the *MAP-kinase cascade*, in which a series of protein kinases phosphorylate and activate one another in sequence (31). Ras activates MAP-kinase-kinase-kinase, which in turn phosphorylates and activates MAP-kinase-kinase, which then activates MAP-kinase. The active MAP-kinase phospholylates different proteins, amongst them extracellular regulated kinase (ERK) into phosphoERK (29). These activated proteins control gene transcription that causes a change of gene expression, thus controlling fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis (32).

The aim of this study

The RAMP group has already done a great deal of experiments on NEAT1_2, particularly on SK-BR-3 cells that are HER2+. This has led to two working theses as a background for my research. The first one is that NEAT1_2 is upregulated by EGF in SK-BR-3 cells, and the upregulation is transient; it peaks after 30 minutes and then decreases again. Earlier experiments also indicates that the upregulation goes through the Ras-MAPK signalling pathway, thus via activation of ERK to phosphorylated ERK. The other work thesis is that the long isoform of NEAT1_2 has a high expression in cells that express HER2, and it looks like there is a positive correlation between HER2+ breast cancers and expression of NEAT1_2.

My aim is to see how pre-treatment with the cancer drugs Gefitinib and Trastuzumab affects expression of NEAT1_2 in both HER2-positive and HER2-negative cells. The hypothesis is that Gefitinib lowers expression of NEAT1_2, because it inhibits the EGF-receptor and thereby also the Ras-MAPK signalling pathway. I've also looked at how treatment with Trastuzumab affects NEAT1_2 expression on HER2+ breast cancer cells. As activation of HER2 can activate several intracellular signalling pathways, including Ras-MAPK, an inhibition with Trastuzumab would be expected to affect both activation of ERK to phosphoERK and expression of NEAT1_2.

The working process

Two weeks during MED-2520, in the autumn of 2014, was used to contact Maria Perander and discuss the potential of making a fifth year assignment a part of their research program. We prepared a project description and she gave me some reading material on subjects that they were working with for preparation.

Two weeks in August 2015 were used to learn how to do proper searching in PubMed, to read through articles relevant to the theme, and to learn how to use EndNote to make a correct list of references.

During the spring of 2016 I've been 9 weeks in the lab, in which the first three was mostly training and learning different methods, and the last six was used to do my experiments. The remaining three weeks of my project period was used to write and submit the thesis.

Relevant materials and methods used in this study

I have worked with two different cell lines, HeLa cells witch are human cervical epithelial cells, and SK-BR-3 cells from human adenocarcinoma in breasts. I have pre-treated them with two different drugs, *Gefitinib* and *Trastuzumab*, and stimulated them with epidermal growth factor (EGF). By RNA isolation and RT-qPCR I've detected the expression of NEAT1_2 relative to GADPH, which is a constitutively expressed housekeeping ncRNA. I've also done Western Blot to detect ERK- and phosphorylated ERK (phosphoERK) proteins, to verify that the EGF succeeded in stimulating the cells though activation of the Ras-MAPK signalling pathway.

Gefitinib and Trastuzumab

Gefitinib is used to treat tumors with activating mutations in the EGF receptor (EGFR) tyrosine kinase domain. It binds to the ATP binding site in the tyrosine kinase domain and function as an ATP competitor, thereby selectively inhibits EGFR. An activating mutation in EGFR is an important factor that promotes tumor growth and inhibition of apoptosis, causes increased production of angiogenetic factors, and facilitates metastatic processes in cancer cells.

Trastuzumab is a recombinant humanized IgG_1 monoclonal antibody against HER2, which binds with high affinity and specificity to its extracellular domain. This leads to inhibition of ligand independent HER2-signalling and prevention of proteolytic cleavage of the extracellular domain, which is an important activation mechanism for HER2. In this way, Trastuzumab inhibits proliferation of human tumor cells with increased expression of HER2, and affect antibody dependent cell mediated cytotoxicity.

RT-qPCR

The amount of gene expressed in a cell can be measured by the number of copies of RNA transcript from that gene present in a sample. The RNA sample is first reverse-transcribed into complimentary DNA (cDNA) by reverse transcriptase. Polymerase chain reaction (PCR) is a common method for amplifying DNA to detect and quantify the expression of genes from small amounts of RNA. Real time quantitative PCR (RT-qPCR) monitors the amplification of a target DNA molecule during the PCR, and not only at the end as with conventional PCR. The same methods are used, with a DNA template, at least one pair of specific primers, deoxyribonucleotides, a suitable buffer solution, and a thermo-stable DNA polymerase.

The concept of RT-qPCR is that amplification of DNA products is measured using a fluorescent label. A fluorescent dye binds directly or indirectly via a probe to the DNA molecules. The fluorescent signal is directly proportional to the concentration of DNA, and the amount of template present in the start of the reaction is calculated by the linear correlation between PCR product and fluorescent intensity. At the point in which fluorescence is first statistically significant detected above a baseline, is called the threshold cycle, or the *Cq value*. This is the most important parameter, as it has to be established to quantify the amount of DNA in the sample. The higher the initial amount of DNA, the sooner the accumulated product is detected, and the lower the Cq value becomes.

The ΔΔCq method is commonly used to quantify data from RT-qPCR. It's done by relative quantification of the gene of interest compared to an endogenous housekeeping gene, which is selected because of its constitutively level of expression. The equation to calculate ΔCq value is: $Cq_{\text{gene of interest}} - Cq_{\text{housekeeping gene}}$, then ΔΔCq is calculated by retracting ΔCq of the zero control from the ΔCq value of the samples of interest: $\Delta\Delta Cq = \Delta Cq_{\text{sample of interest}} - \Delta Cq_{\text{zero control}}$. From the ΔΔCq it's possible to calculate fold change, which describes how much a quantity doubles from initial to final value, the equation being $2^{-\Delta\Delta Cq}$.

Western blot

Western blot is used to detect specific proteins in a sample. The first step is gel electrophoresis, where denatured proteins are separated by polypeptide length, sometimes in combination with electrical charge. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most common type, which maintains polypeptides in a denatured state after treatment with strong reducing agents to remove secondary and tertiary structures. This allows separation based on molecular weight. The proteins are covered in negatively charged SDS, resulting in movement towards the positively charged electrode, through the acrylamide mesh of the gel. Small proteins move faster than the larger once, thus are the proteins separated based on size. Samples are loaded into wells, and one of the lanes are usually reserved to a marker or a ladder that is a mixture of proteins with defined molecular weights, and are stained to form visible bands.

Afterwards the proteins are transferred to a membrane, maintaining the same organization as in the gel, to become accessible to antibody detection. The membrane has none-specific

protein binding properties, meaning that it binds all proteins equally well. The next step is blocking the membrane to avoid interactions between the membrane and the antibody used to detect target protein. Soaking the membrane in a blocking solution makes sure that proteins bind to all places that target proteins haven't attached, so there is no room on the membrane for the antibody to attach when it's added.

After blocking, the membranes are subjected to a dilute solution with primary antibody and incubated with gentle agitation. Then the membrane is rinsed to remove unattached primary antibody, and next subjected to secondary antibody directed at a species-specific part of the primary antibody. There are different types of secondary antibodies, and therefore different ways of visualising the target proteins on the membrane. A precise way of quantifying the amount of protein in a sample is by using a near-infrared fluorophore-linked antibody. Light from excitation of a fluorescent dye is static, enabling an accurate measure of the difference in signal produced by labelled antibodies bound to proteins on the western blot. A photosensor equipped with appropriate emission filters captures a digital image of the blot, which allows further analysis; such as molecular weight analysis and quantitative western blot analysis.

Methods

The same methods, RT-qPCR and western blot, are used for three different experiments; 1) HeLa cells pre-treated with Gefitinib, 2) SK-BR-3 cells pre-treated with Gefitinib and finally 3) SK-BR-3 cells pre-treated with Trastuzumab. Both cell lines express EGF receptors, hence are both treated with Gefitinib, whereas only the SK-BR-3 cells express HER2 and were pre-treated with Trastuzumab.

Cell Cultures

The reason for choosing HeLa cells and SK-BR-3 cells is that HeLa cells are stable and easy to work with, so it's suitable to use when learning how to culture cells. They also function as a comparison to SK-BR- 3 cells that are HER2+. The RAMP group has already used SK-BR-3 cells to a great extent to do NEAT1_2 experiments, hence they gave rise to my work theses.

HeLa- and SK-BR-3 cells were purchased from the American Type Culture Collection (ATCC). HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich), supplemented with 10 % Fetal Bovine Serum (FBS) (Biochrom) and 1 % penicillin-streptomycin (Sigma Aldrich). SK-BR-3 were cultured in RPMI 1640 (Sigma Aldrich), also supplemented with 10 % Fetal Bovine Serum (FBS) (Biochrom) and 1 % penicillin-streptomycin (Sigma Aldrich). Both cell lines were incubated in a 5 % CO_2 humidified incubator at 37 °C.

Cellular treatments

A bürker chamber was used to count the cells, and approximately 100 000 cells per well were transferred into 48 different wells (12 well plate) for each experiment to be able to make triplets for every cell condition. They were further incubated in a 5 % CO_2 humidified incubator at 37°C, the HeLa cells for 18 hours and SK-BR-3 cells for 72 hours. Next, they were starved for at least 18 hours in media only containing 0.5% serum.

For the two experiments with Gefitinib, Gefitinib (diluted to 10 mg/ml in Dimethyl Sulphoxide (DMSO)), was added to half of the cell wells (G+) to give a final concentration of 450 ng/ml. A control of equal concentration of DMSO diluted in H_2O was added to the other half (G-). For the third and last experiment, Trastuzumab (hospital pharmacy, UNN) was diluted in H_2O to a concentration of 20 mg/ml and were added to half of the cell wells (T+), giving a final concentration of 10 µg/ml. A control with an equal volume of H_2O were added to the other half (T-). After two hours the cells were stimulated with EGF (Sigma-Aldrich) to a final concentration of 20 ng/ml for each well. Afterwards they were washed with ice cold PBS (Sigma Aldrich) and harvested at four different time points after EGF-stimulation; 0 minutes (no EGF added), 30 minutes, 1 hour and 2 hours.

Preparation of cellular extracts to RT-qPCR and RNA isolation

While harvesting, 250 µl TRIzol Reagent (ThermoFisher Sientific) was added to the samples that were later used for RNA isolation to lysate the cells. The dish was pipetted up and down several times and transferred to clean tubes. After 5 minutes in room temperature the RNA-samples were incubated at -70 °C until further use.

Isolation of RNA was done over two days and the whole process was done in compartment lockers. The first step on day one was *Phase separating*. 0.2 volumes of Chloroform were added to the RNA-samples and they were shaken thoroughly, before placing them on ice in 20 minutes. Next they were centrifuged at 9 000 rpm for 20 minutes at 4 °C. Then the lower phase with proteins and DNA was removed, and the samples were centrifuged again at 9 000 rpm for another 20 minutes at 4 °C. Afterwards the aqueous phase with the RNA was transferred to a new clean tube. The next step on day one was *RNA precipitation.* 1 volume of Isopropanol was added to the samples and they were incubated at -20 °C over night.

RNA precipitation continued on day two with centrifugation at 15 000 rpm for 30 minutes at 4 °C. After centrifugation the supernatant was discarded, and the samples were washed with 1 ml 80 % EtOH and then centrifuged again at 13 000 rpm for 5 minutes in room temperature. The EtOH was removed before another round of centrifugation at 13 000 rpm for only 1 minute in room temperature. The rest of the EtOH was removed and the tubes were left open to air-dry the RNA pellet for 5-10 minutes. Finally the RNA pellet was resuspended in 15 µl RNase free H_2O. The samples were then kept at -70 °C until they were counted and used to cDNA synthesis.

cDNA Synthesis and RT-qPCR

The RNA samples were counted using Nano Drop 2000c (Thermo Scientific) before reverse transcriptase PCR was done to produce cDNA from RNA. To do a 10 µl cDNA reaction, RNA samples were diluted in RNase free H_2O to volumes of 4.5 µl. The HeLa cells pre-treated with Gefitinib consisting of 250 ng per sample, the SK-BR-3 cells pre-treated with Gefitinib had 150 ng RNA per sample, and finally the SK-BR-3 cells pre-treated with Trastuzumab containing 500 ng RNA per sample. A mastermix with 0.5 µl random hexamer primer (100 µM) (ThermoFisher Sientfic) and 2 µl dNTPs (2.5 µM each) were added to each of the RNA samples, resulting in a volume of 7 µl. Next the samples were heated at at 65 °C for 5 minutes and cooled down again at 4 °C for at least 1 minute. A second mastermix consisting of 2 µl 5X FS buffer (ThermoFisher Sientific), 0.5 µl DTT (0.1 M) (ThermoFisher Sientific) and 0.5 µl SuperScript™ III Reverse Transcriptase (ThermoFisher Sientific) were next added to each sample, to the final volume of 10 µl. GeneAmp® PRC system 9700 was used to do the PCR reaction with 25 °C for 5 minutes, 50 °C for 60 minutes and 70 °C for 15 minutes. Afterwards the cDNA were kept at 4 °C until RT-qPCR were done.

cDNA were diluted in H_2O, 1:5 for the SK-BR-3 cells +/- Gefitinib that consisted of only 150 ng RNA in the output sample, and 1:10 for the other samples (HeLa +/- Gefitinib and SK-BR-3 +/- Trastuzumab). Two new mastermixes were made of 5 µl FastStart Essential DNA Green Master (Roche Life Sience) and 2.5 µl primer sets, one for NEAT1_2 and one for GAPDH. 7.5 µl mastermix with each of the primer sets were transferred to wells on a qPCR plate. 2.5 µl diluted cDNA was added, resulting in cDNA from every sample mixed in one well with primers for NEAT1_2 and in another well for with primers for GAPDH. A control from the cDNA synthesis and a new control of 10 µl for the qPCR were also included on the plate. The LightCycler® 96 was used for quantification and the ΔΔCq-method was used to calculate fold change using GAPDH as internal reference.

SDS-PAGE and Western Blot

100 µl Loading Buffer was added to the samples that were used to do SDS-PAGE and Western blot for manufacturing proteins. The Loading Buffer consisted of 50 % NuPAGE® LDS Sample Buffer (4X) (Thermo Fisher Scientific), 20 % NuPAGE®Sample Reducing Agent (10X) (Thermo Fisher Scientific), and 30 % H_2O to a 2X solution. The protein sample dish was resuspended a few times before transferring to clean tubes, and then heated at 70 °C in 10 minutes, then incubated at -20 °C until further use.

SDS-PAGE and Western blotting were performed following the NuPAGE Technical Guide (IM9051, Invitrogen instruction manual, Revised 2009). The protein samples were heated for 10 minutes at 70 °C, and then sonicated briefly to make sure the cells were completely lysed. SDS-PAGE was prepared by washing the wells of NuPAGE® Novex 4-12% Bis-Tris Gels (Invitrogen) with NuPAGE® MES SDS Running Buffer (ThermoFisher Scientific). 200 ml running buffer with 500 µl antioxidant were added to the inner chamber and 600 ml running buffer were added to the outer chamber. 3 µl MagicMark™ XP Western Protein Standard (ThermoFisher Scientific) and 5 µl SeaBlue® Plus2 PreStained Protein Standard (ThermoFisher Scientific) was used as ladders, and 20 µl sample were spread through the wells of the gel. SDS-PAGE was run for 40 minutes with a constant of 200 V.

The blotting of the proteins onto a nitrocellulose membrane was performed at 30 V in 90 minutes as described in XCell II Blot Module (Invitrogen Instruction Manual, IM9051).

Filter papers, membrane and pads were fist soaked in *transfer buffer*, and put together in the inner chamber in this order; pad-pad-filter paper-gel-membrane-filter paper-pad-pad. The inner chamber was filled with transfer buffer, and the outer chamber with MqH_2O.

After the blot the membrane was incubated in Odyssey® Blocking Buffer (Li-Cor) in room temperature with shaking for approximately 1 hour. Primary antibodies against ERK1/-2 or phospho-ERK1/-2 (Cell Signaling Technology) were diluted in the NuPAGE Blocking Buffer with 0.1% Tween and were incubated with the membranes overnight at 4 °C. Next the membranes were washed with 1x TBS-T 6 times for 5 minutes, before it was probed with secondary antibody IRDye®800CW Goat anti-Rabbit (Li-Cor) and IRDye®800CW Goat anti-Mouse (Li-Cor) (1:7000) for ERK1/-2- or phosphoERK1/-2 antibodies respectively, incubated in 1 hour. The membranes were wash again 6 times with 1x TBS-T before they were visualized using the Odyssey IR Imaging System (Li-COR).

Results

NEAT1_2 is induced by EGF in HeLa cells

The RAMP research group has previously shown that NEAT1_2 expression in SK-BR-3 was induced upon EGF treatment. We wanted to see if EGF also induced NEAT1_2 in HeLa cells that express EGFR, but not the HER2 receptor. HeLa cells stimulated with EGF, showed a transient induction in NEAT1_2 expression (*figure 4)* as determined by RT-qPCR. There was a boost in expression 30 minutes after EGF stimulation that decreased again after 1 hour. We pre-treated the cells with Gefitinib that inhibits the EGFR. Gefitinib did not abolish the EGF-mediated NEAT1_2 expression, as it was as much as eight times greater for the cells exposed to Gefitinib, whereas the control showed a lesser increase with a fold change of approximately 5. It was only a slight difference in NEAT1_2 expression with no EGF stimulation between untreated cells and cells treated with drug.

Gefitinib transiently inhibited activation of ERK in HeLa cells treated the same way (*figure 5*). There were quite similar amounts of phosphorylated ERK (pERK) both with and without drug treatment before EGF stimulation. Thirty minutes after stimulation there was a clear difference between untreated cells and cells where the EGF receptor was inhibited by

Gefitinib. The effect of EGF decreased after 30 minutes and the amount of phosphorylated ERK was even bigger with drug treatment when time reached one- and two hours.

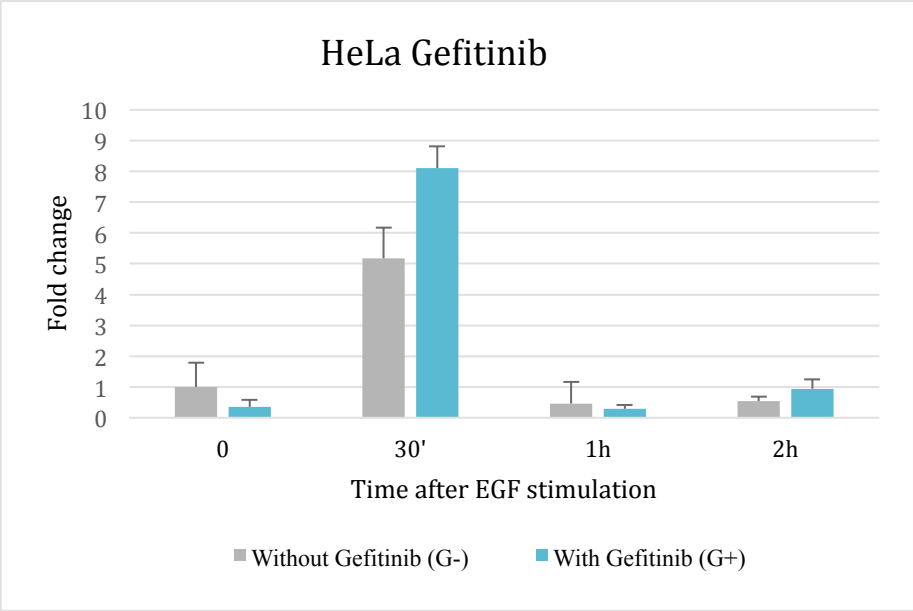

Figure 4: NEAT1_2 is induced by EGF in HeLa cells. Untreated or Gefitinib treated HeLa cells were stimulated with EGF for the indicated time periods. RT-qPCR analyses were performed using primers recognizing NEAT1_2. The results are mean values of biological triplicates in one experiment and fold changes relative to unstimulated cells are shown.

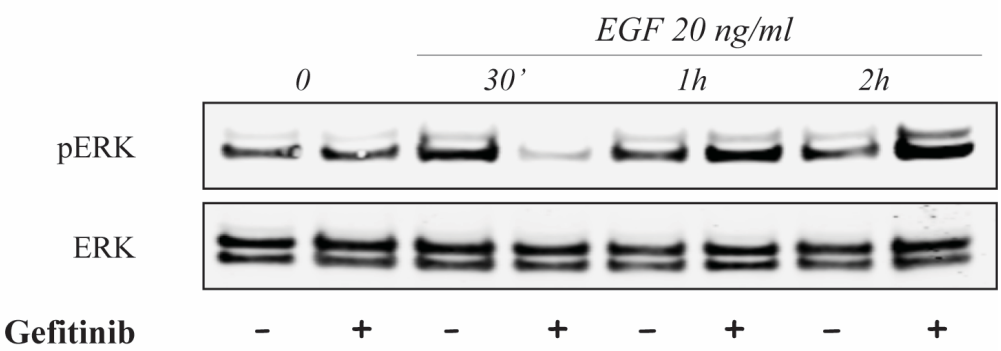

Figure 5: Gefitinib transiently inhibits EGF-mediated ERK1/-2 activation in HeLa cells. HeLa cells were treated as in figure 4 and proteins were harvested at indicated time points. Western blot were perform using antibody recognizing phosphorylated ERK1/-2 (pERK). Equal loading of proteins was verified by blotting against total ERK1/-2 (ERK).

Gefitinib inhibits EGF-mediated NEAT1_2 induction in SK-BR-3 cells

We next set out to analyse the effect of Gefitinib on EGF-mediated NEAT1_2 induction in SK-BR-3 cells. SK-BR-3 stimulated with EGF also showed a transient induction in NEAT1_2 expression (*figure 6*). 30 minutes after stimulation with EGF without Gefitinib pre-treatment the expression increased to a fold change of 4, whereas it was virtually

unchanged from 0 to 30 minutes in the cells where the EGF receptor was inhibited. Expression then decreased one- and two hours after EGF stimulation. Worth noticing is the extremely high standard deviations 30 minutes and one hour after EGF stimulation without drug treatment.

Gefitinib inhibited EGF-mediated activation of total ERK in SK-BR-3 cells (*figure 7*). The amount of phosphorylated ERK (pERK) is higher in samples from untreated cells than cells exposed to pre-treatment with Gefitinib at all indicated time points.

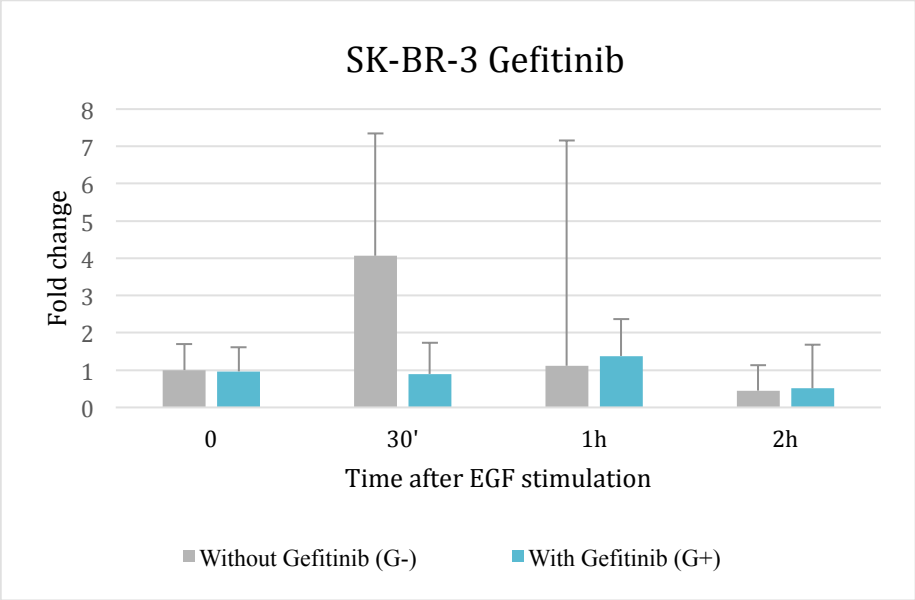

Figure 6: Gefitinib inhibits EGF-mediated NEAT1_2_induction in SK-BR-3 cells. Untreated or Gefitinib treated SK-BR-3 cells were stimulated with EGF for the indicated time periods. RT-qPCR analyses were performed using primers recognizing NEAT1_2. The results are mean values of biological triplicates in one experiment and fold changes relative to unstimulated cells are shown.

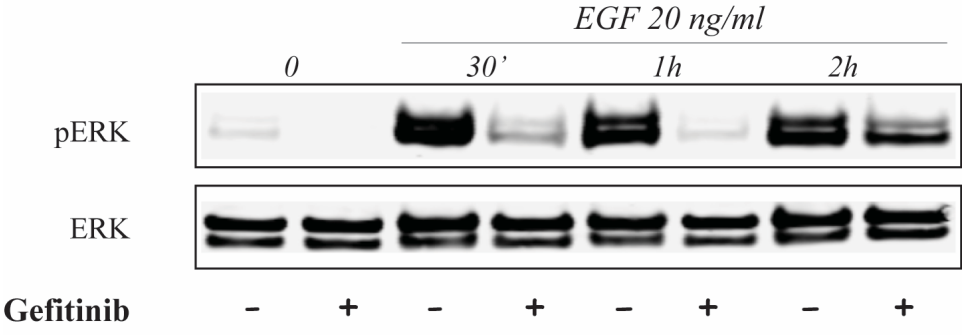

Figure 7: Gefitinib inhibits EGF-mediated ERK1/-2 activation in SK-BR-3 cells. SK-BR-3 cells were treated as in figure 6 and proteins were harvested at indicated time points. Western blot were perform using antibody recognizing phosphorylated ERK1/-2 (pERK). Equal loading of proteins was verified by blotting against total ERK1/-2 (ERK).

Trastuzumab reduces EGF-mediated NEAT1_2 induction in SK-BR-3 cells

We next studied the effect of Trastuzumab on NEAT1_2 induction in SK-BR-3 cells. SK-BR-3 cells stimulated with EGF showed an induction in NEAT1_2 expression (*figure 8*). The baseline activation of the cells was smaller in this experiment compared to the one with SK-BR-3 cells and Gefitinib, as the highest fold change of non-treated cells was 4 in figure 3, whereas it barely reached 2.5 under the same conditions in this experiment. However, there was a transient induction in NEAT1_2 expression without drug treatment, as it increased after 30 minutes, and then gradually decreased again after one- and two hours. Inhibition of HER2 by pre-treatment with Trastuzumab lowered NEAT1_2 expression 30 minutes- and one hour after EGF stimulation compared to untreated cells.

Trastuzumab did not inhibit activation of ERK in SK-BR-3 cells (*figure 9*). Almost equal amounts of phosphorylated ERK are found in the samples from the same time points both with and without drug pre-treatment.

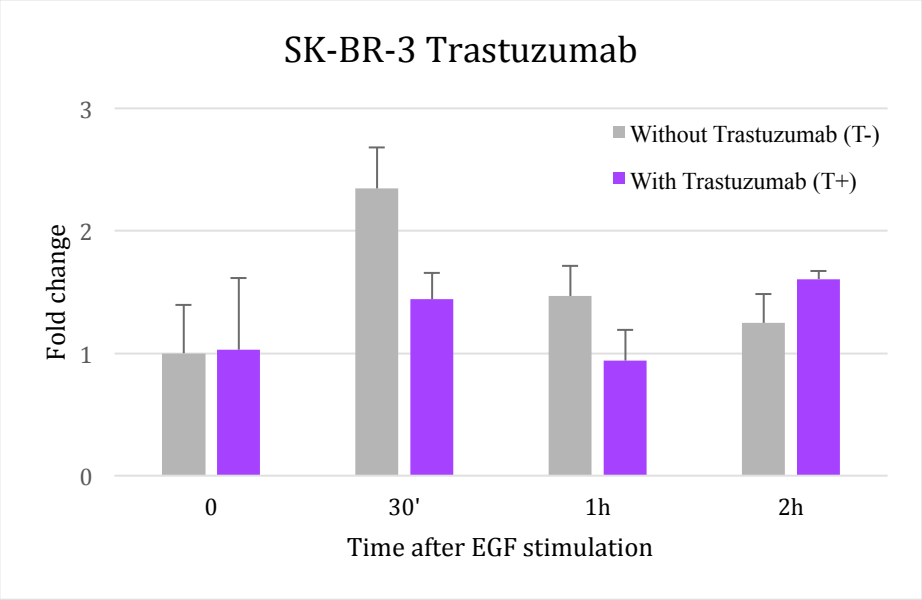

Figure 8: Trastuzumanb reduces EGF-mediated NEAT1_2 induction in SK-BR-3 cells.. Untreated or Trastuzumab treated SK-BR-3 cells were stimulated with EGF for the indicated time periods. RT-qPCR analyses were performed using primers recognizing NEAT1_2. The results are mean values of biological triplicates in one experiment and fold changes relative to unstimulated cells are shown. Notice the difference in the scale used on the y-axis, reflecting fold change, used in this figure (0-3) compared to SK-BR-3 cells pre-treated with Gefitinib (0-8).

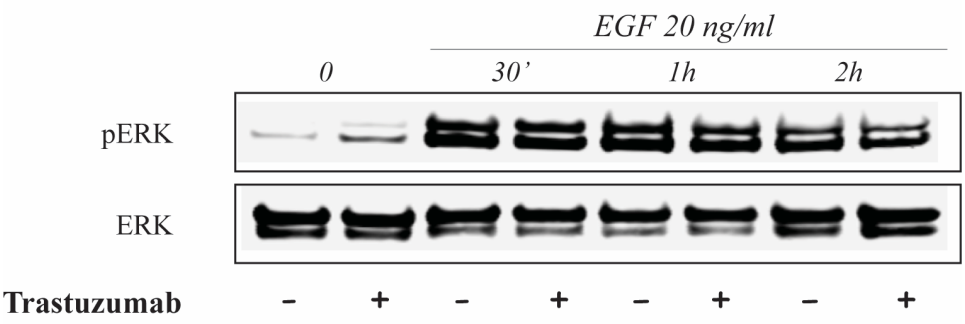

Figure 9: Trastuzumab does not inhibit EGF-mediated ERK1/-2 activation in SK-BR-3 cells. SK-BR-3 cells were treated as in figure 8 and proteins were harvested at indicated time points. Western blot were perform using antibody recognizing phosphorylated ERK1/-2 (pERK). Equal loading of proteins was verified by blotting against total ERK1/-2 (ERK).

Discussion

Gefitinib did not work quite as expected on HeLa cells for unknown reasons. Pre-treatment with Gefitinib succeeded in inhibiting EGF-stimulated activation of ERK into phosphoERK on HeLa cells 30 minutes after stimulation. Surprisingly, the expression of NEAT1_2 seems to be highest under the same conditions. This would not be expected if NEAT1_2 expression is upregulated by activation with EGF, as believed for SK-BR-3 cells. It appears that the effect of Gefitinib on HeLa cells decreases after 30 minutes, and somewhat unexpected, the amount of phosphoERK seems to be even bigger with drug treatment than without when time reaches one- and two hours.

In the experiment with Gefitinib and SK-BR-3 cells there was a transient induction of NEAT1_2 as expected. Inhibition of the EGF receptor prevented this induction in cells that were pre-treated with Gefitinib. This corresponds to the working thesis that NEAT1_2 is transiently upregulated by EGF in SK-BR-3 cells. The large standard deviations 30 minutes and one hour after EGF stimulation without drug treatment is because there is one very anomalous result within the triplets for these samples (see Appendix I, raw data from RT-qPCR results). Reproduction of the experiment should be conducted to increase the number of replicates. Gefitinib succeeded very well in inhibiting EGF-mediated activation of ERK into phosphorylated ERK as expected, shown by the western blot in figure 7.

SK-BR-3 cells pre-treated with Trastuzumab and stimulated with EGF show a light induction in NEAT1_2 expression. SK-BR-3 cells are HER2-positive, and activation of HER2 can activate the Ras-MAPK signalling pathway. In addition to this, indications point at correlation between HER2-positive breast cancers and expression of NEAT1_2. This led to the hypothesis that inhibition of HER2 with Trastuzumab would lower NEAT1_2 expression. From the western blot in figure 9 it looks like inhibition of HER2 is not sufficient to inhibit activation of ERK into phosphoERK, thus if induction of NEAT1_2 goes through activation of the Ras-MAPK signalling pathway, Trastuzumab would not be able to inhibit NEAT1_2 induction. However, we did observed a slight reduction in EGF-mediated NEAT1_2 expression in cells pre-treated with Trastuzumab.

As my experiments are done as a pilot study and are only performed once, it is impossible to draw any conclusions with statistical significance from my work. Standard deviations between samples form cells treated under the same conditions are also too high to rely on, especially in the experiment with SK-BR-3 cells and Gefitinib. Even though the results would have to be reproduced in order to conclude, they can still be used to give rise to new working theses on how the mysterious lncRNA NEAT1_2 play a role in breast cancer.

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Appendix I: Raw data from laboratory experiments

Nano Drop RNA count

HeLa and Gefitinib

HeLa Gef #	Sample ID	Nucleic Acid	Unit	260/280	260/230	RNA 250 ng	H2O
1	EGF0 G+ R1	134	ng/µl	1,8	1,62	1,9	2,6
2	EGF0 G+ R2	113,8	ng/µl	1,82	1,06	2,2	2,3
3	EGF0 G+ R3	121,6	ng/µl	1,8	1,42	2,1	2,4
4	EGF0 G- R1	191,5	ng/µl	1,81	2,02	1,3	3,2
5	EGF0 G- R2	118,2	ng/µl	1,79	2,11	2,1	2,4
6	EGF0 G- R3	178,9	ng/µl	1,84	1,71	1,4	3,1
7	EGF30 G+ R1	0,2	ng/µl	-0,39	-0,79	-	-
8	EGF30 G+ R2	235,6	ng/µl	1,79	2,18	1,1	3,4
9	EGF30 G+ R3	246,2	ng/µl	1,72	0,62	1,0	3,5
10	EGF30 G- R1	194,8	ng/µl	1,76	1,29	1,3	3,2
11	EGF30 G- R2	211,9	ng/µl	1,74	0,94	1,2	3,3
12	EGF30 G- R3	205,3	ng/µl	1,8	1,35	1,2	3,3
13	EGF1 G+ R1	130,3	ng/µl	1,78	1,88	1,9	2,6
14	EGF1 G+ R2	94,9	ng/µl	1,75	1,71	2,6	1,9
15	EGF1 G+ R3	138,1	ng/µl	1,76	1,76	1,8	2,7
16	EGF1 G- R1	199,3	ng/µl	1,86	1,97	1,3	3,2
17	EGF1 G- R2	152	ng/µl	1,73	2,18	1,6	2,9
18	EGF1 G- R3	118	ng/µl	1,78	1,67	2,1	2,4
19	EGF2 G+ R1	71,4	ng/µl	1,81	1,25	3,5	1,0
20	EGF2 G+ R2	56	ng/µl	1,75	1,57	4,5	0,0
21	EGF2 G+ R3	58,2	ng/µl	1,7	2,08	4,3	0,2
22	EGF2 G- R1	65,6	ng/µl	1,79	1,35	3,8	0,7
23	EGF2 G- R2	176,3	ng/µl	1,8	2,06	1,4	3,1
24	EGF2 G- R3	178,8	ng/µl	1,8	2,3	1,4	3,1

SK-BR-3 and Gefitinib

SKBR3 Gef #	Sample ID	Nucleic Acid	Unit	260/280	260/230	RNA 150 ng	H2O
1	EGF0 G+ R1	75,5	ng/µl	1,79	1,53	2,0	2,5
2	EGF0 G+ R2	25,7	ng/µl	1,7	0,46	5,8	-1,3
3	EGF0 G+ R3	41,8	ng/µl	1,75	1,26	3,6	0,9
4	EGF0 G- R1	43	ng/µl	1,67	0,71	3,5	1,0
5	EGF0 G- R2	56,4	ng/µl	1,7	1,35	2,7	1,8
6	EGF0 G- R3	89	ng/µl	1,78	1,36	1,7	2,8
7	EGF1 G+ R1	48	ng/µl	1,89	0,27	3,1	1,4
8	EGF1 G+ R2	57,8	ng/µl	1,74	1,95	2,6	1,9
9	EGF30 G+ R3	119,7	ng/µl	1,78	1,78	1,3	3,2
10	EGF30 G- R1	131,8	ng/µl	1,82	1,78	1,1	3,4
11	EGF30 G- R2	94,6	ng/µl	1,73	1,31	1,6	2,9
12	EGF30 G- R3	105,4	ng/µl	1,83	1,73	1,4	3,1
13	EGF1 G+ R1	80	ng/µl	1,73	2,25	1,9	2,6
14	EGF1 G+ R2	75,5	ng/µl	1,78	1,45	2,0	2,5
15	EGF1 G+ R3	68	ng/µl	1,78	1,04	2,2	2,3
16	EGF1 G- R1	59,3	ng/µl	1,8	0,6	2,5	2,0
17	EGF1 G- R2	154,2	ng/µl	1,8	1,94	1,0	3,5
18	EGF1 G- R3	52	ng/µl	1,87	0,09	-	-
19	EGF2 G+ R1	61,4	ng/µl	1,73	1,69	2,4	2,1
20	EGF2 G+ R2	54,6	ng/µl	1,81	1,4	2,7	1,8
21	EGF2 G+ R3	152,4	ng/µl	1,83	1,8	1,0	3,5
22	EGF2 G- R1	128,7	ng/µl	1,86	0,86	1,2	3,3
23	EGF2 G- R2	148,6	ng/µl	1,83	1,95	1,0	3,5
24	EGF2 G- R3*	30,3	ng/µl	1,75	1,21	5,0	-0,5

SK-BR-3 and Trastuzumab

SKBR3 T #	Sample ID	Nucleic Acid	Unit	260/280	260/230	500 ng RNA	H2O
1	EGF0 T+ R1	243,9	ng/µl	1,84	2,29	2,1	2,4
2	EGF0 T+ R2	456,3	ng/µl	1,86	2,22	1,1	3,4
3	EGF0 T+ R3	271,3	ng/µl	1,84	2,28	1,8	2,7
4	EGF0 T- R1	296,7	ng/µl	1,83	2,3	1,7	2,8
5	EGF0 T- R2	276	ng/µl	1,87	2,09	1,8	2,7
6	EGF0 T- R3	299,4	ng/µl	1,84	2,33	1,7	2,8
7	EGF30 T+ R1	362,2	ng/µl	1,86	2,24	1,4	3,1
8	EGF30 T+ R2	425,5	ng/µl	1,86	2,27	1,2	3,3
9	EGF30 T+ R3	266,1	ng/µl	1,84	2,28	1,9	2,6
10	EGF30 T- R1	437,1	ng/µl	1,87	2,24	1,1	3,4
11	EGF30 T- R2	503,4	ng/µl	1,88	2,29	1,0	3,5
12	EGF30 T- R3	501,9	ng/µl	1,84	2,24	1,0	3,5
13	EGF1 T+ R1	276,3	ng/µl	1,84	2,26	1,8	2,7
14	EGF1 T+ R2	249,4	ng/µl	1,78	2,39	2,0	2,5
15	EGF1 T+ R3	227,9	ng/µl	1,85	2,24	2,2	2,3
16	EGF1 T- R1	387,1	ng/µl	1,83	2,33	1,3	3,2
17	EGF1 T- R2	457,7	ng/µl	1,86	2,25	1,1	3,4
18	EGF1 T- R3	451,9	ng/µl	1,86	2,26	1,1	3,4
19	EGF2 T+ R1	568,9	ng/µl	1,9	2,36	0,9	3,6
20	EGF2 T+ R2	620,4	ng/µl	1,89	2,3	0,8	3,7
21	EGF2 T+ R3	294,4	ng/µl	1,83	2,35	1,7	2,8
22	EGF2 T- R1	615,6	ng/µl	1,89	2,34	0,8	3,7
23	EGF2 T- R2	443	ng/µl	1,85	2,29	1,1	3,4
24	EGF2 T- R3	640,4	ng/µl	1,87	2,32	0,8	3,7

RT-qPCR

HeLa and Gefitinib

Position	Sample	Cq GAPDH	Cq NEAT1.2	Delta ct	Mean	Delta delta ct	Fold change	Mean FC	STD.AV
1	EGF0 G- R1	16,21	27,09	10,88	11,83	-0,95	1,94	1,13	0,70
2	EGF0 G- R2	16,02	28,21	12,19		0,36	0,78		
3	EGF0 G- R3	16,54	28,97	12,43		0,60	0,66		
4	EGF0 G+ R1	15,98	30,08	14,10		2,27	0,21	0,38	0,16
5	EGF0 G+ R2	17,01	30,12	13,11		1,28	0,41		
6	EGF0 G+ R3	16,27	29,03	12,76		0,93	0,53		
7	EGF30' G- R1	16,55	-	-	-	-	-	-	-
8	EGF30' G- R2	16,37	25,65	9,28		-2,55	5,87		
9	EGF30' G- R3	16,41	26,05	9,64		-2,19	4,57		
10	EGF30' G+ R1	-	-	-	-	-	-	-	-
11	EGF30' G+ R2	16,63	25,53	8,90		-2,93	7,64	8,12	0,68
12	EGF30' G+ R3	16,02	24,75	8,73		-3,10	8,59		
13	EGF1h G- R1	16,38	30,85	14,47		2,64	0,16	0,58	0,37
14	EGF1h G- R2	16,26	28,3	12,04		0,21	0,87		
15	EGF1h G- R3	16,10	28,43	12,33		0,50	0,71		
16	EGF1h G+ R1	16,24	29,76	13,52		1,69	0,31	0,30	0,10
17	EGF1h G+ R2	16,30	30,48	14,18		2,35	0,20		
18	EGF1h G+ R3	16,40	29,59	13,19		1,36	0,39		
19	EGF2h G- R1	16,37	28,71	12,34		0,51	0,70	0,55	0,14
20	EGF2h G- R2	16,24	29,28	13,04		1,21	0,43		
21	EGF2h G- R3	15,96	28,74	12,78		0,95	0,52		
22	EGF2h G+ R1	16,10	27,55	11,45		-0,38	1,30	0,97	0,29
23	EGF2h G+ R2	16,55	28,69	12,14		0,31	0,81		
24	EGF2h G+ R3	16,57	28,73	12,16		0,33	0,80		
	Ctr 1	35,34	36,47	1,13		-10,70	-		
	Ctr2	0,00	0	0,00		-11,83	-		

SK-BR-3 and Gefitinib

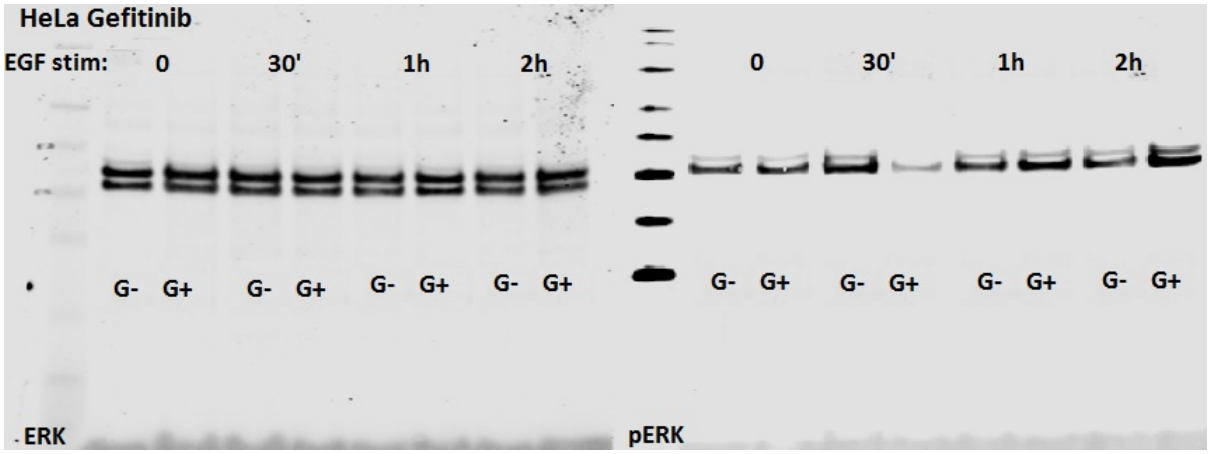
Position	Sample	Cq GAPDH	Cq NEAT1.2	Delta ct	Mean	Delta delta ct	Fold change	Mean FC	STD.AV
1	EGF0 G- R1	-	27,02	-	-	-	-	-	-
2	EGF0 G- R2	15,91	29,27	13,36	12,82	0,55	1,46	1,07	0,55
3	EGF0 G- R3	15,47	27,74	12,27		-0,55	0,69		
4	EGF0 G+ R1	15,20	28,75	13,55		0,74	1,66	1,12	0,53
5	EGF0 G+ R2	15,48	27,55	12,07		-0,74	0,60		
6	EGF0 G+ R3	16,20	29,17	12,97		0,16	1,11		
7	EGF30' G- R1	15,53	25,84	10,31		-2,51	0,18	0,28	0,18
8	EGF30' G- R2	15,62	27,40	11,78		-1,04	0,49		
9	EGF30' G- R3	15,26	25,55	10,29		-2,53	0,17		
10	EGF30' G+ R1	15,16	28,54	13,38		0,57	1,48	1,26	0,66
11	EGF30' G+ R2	15,21	28,86	13,65		0,84	1,78		
12	EGF30' G+ R3	15,05	26,93	11,88		-0,93	0,52		
13	EGF1h G- R1	15,23	25,99	10,76		-2,06	0,24	1,78	2,18
14	EGF1h G- R2	15,17	29,72	14,55		1,74	3,33		
15	EGF1h G- R3	-	-	-	-	-	-		
16	EGF1h G+ R1	15,56	28,63	13,07		0,26	1,19	0,79	0,39
17	EGF1h G+ R2	15,92	28,37	12,45		-0,36	0,78		
18	EGF1h G+ R3	15,05	26,58	11,53		-1,29	0,41		
19	EGF2h G- R1	15,58	28,81	13,23		0,42	1,33	3,01	2,96
20	EGF2h G- R2	15,43	28,60	13,17		0,36	1,28		
21	EGF2h G- R3	15,39	30,89	15,50		2,69	6,43		
22	EGF2h G+ R1	15,40	27,98	12,58		-0,23	0,85	3,18	3,78
23	EGF2h G+ R2	15,36	28,38	13,02		0,21	1,15		
24	EGF2h G+ R3	15,32	31,05	15,73		2,92	7,54		
26	Ctr 1	34,64	0	-34,64		-47,455	5,18348E-15		
27	Ctr 2	30,75	0	-30,75		-43,565	7,68472E-14		

SK-BR-3 and Trastuzumab

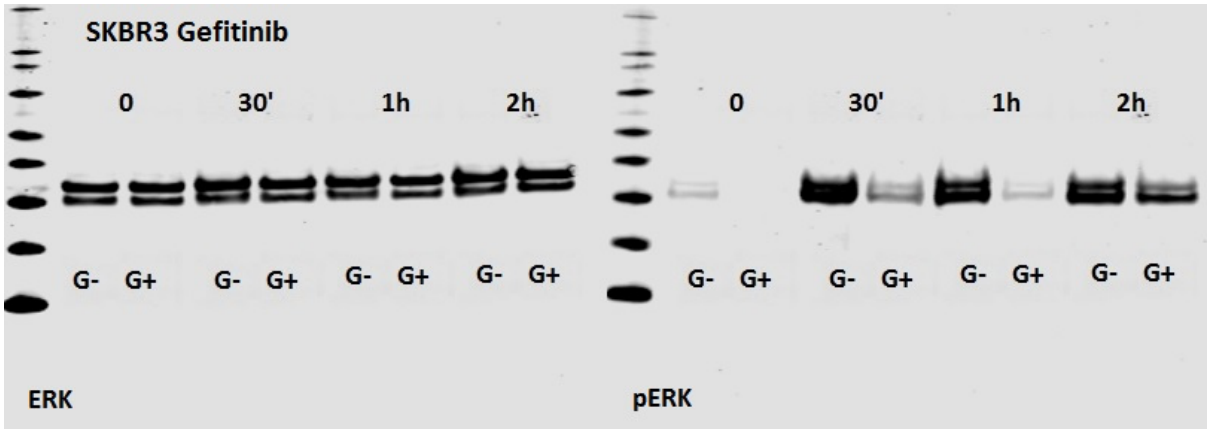
Position	Sample	Cq GAPDH	Cq NEAT1.2	Delta ct	Mean	Delta delta ct	Fold change	Mean FC	STD.AV
1	EGF0 T- R1	14,54	22,24	7,70	8,14	-0,44	0,74	1,04	0,35
2	EGF0 T- R2	14,04	22,69	8,65		0,51	1,43		
3	EGF0 T- R3	14,03	22,09	8,06		-0,08	0,95		
4	EGF0 T+ R1	13,16	21,90	8,74		0,60	1,52	1,04	0,45
5	EGF0 T+ R2	14,65	22,09	7,44		-0,70	0,62		
6	EGF0 T+ R3	14,18	22,29	8,11		-0,03	0,98		
7	EGF30' T- R1	15,30	21,99	6,69		-1,45	0,37	0,43	0,06
8	EGF30' T- R2	15,01	22,07	7,06		-1,08	0,47		
9	EGF30' T- R3	15,21	22,18	6,97		-1,17	0,45		
10	EGF30' T+ R1	14,38	22,22	7,84		-0,30	0,81	0,70	0,10
11	EGF30' T+ R2	14,30	21,80	7,50		-0,64	0,64		
12	EGF30' T+ R3	14,10	21,59	7,49		-0,65	0,64		
13	EGF1h T- R1	14,89	22,36	7,47		-0,67	0,63	0,69	0,11
14	EGF1h T- R2	14,52	22,36	7,84		-0,30	0,81		
15	EGF1h T- R3	15,12	22,56	7,44		-0,70	0,62		
16	EGF1h T+ R1	14,03	22,48	8,45		0,31	1,24	1,09	0,24
17	EGF1h T+ R2	14,03	22,44	8,41		0,27	1,21		
18	EGF1h T+ R3	14,30	22,13	7,83		-0,31	0,81		
19	EGF2h T- R1	15,67	23,21	7,54		-0,60	0,66	0,81	0,13
20	EGF2h T- R2	14,91	22,80	7,89		-0,25	0,84		
21	EGF2h T- R3	15,45	23,47	8,02		-0,12	0,92		
22	EGF2h T+ R1	15,40	22,79	7,39		-0,75	0,60	0,62	0,02
23	EGF2h T+ R2	15,80	23,30	7,50		-0,64	0,64		
24	EGF2h T+ R3	14,81	22,28	7,47		-0,67	0,63		
25	CTR1	-	-						
26	CTR2	-	-						

Western blot

HeLa and Gefitinib



SK-BR-3 and Gefitinib



SK-BR-3 and Trastuzumab