Gene expression of MAP2K1 and Cyclin D1 in BDII rat model of Endometrial cancer

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Supervisor: Neha Singh (neha.singh@his.se)
Supervisor: Anna Pfister (anna.pfister@his.se)
Examiner: Linda Handlin (linda.handlin@his.se)

Almir Budnjo
a12almbu@student.his.se

School of Bioscience
University of Skövde
PO BOX 408
SE-541 28 Skövde
Sweden
Abstract

Endometrial adenocarcinoma (EAC) is the most frequently diagnosed gynecological cancer of the female genital tract in the Western world. Research studies in EC is difficult to conduct on human tumor samples due to the complex nature of tumor arousal and genetic heterogeneousness in the human population. Therefore, inbred animal models can be promising tools to use in EC research due to similar histopathology and pathogenesis as humans. Studies performed on MAP2K1 and CCND1 has shown that their altered expression play a crucial role in carcinogenesis. CCND1 has been demonstrated to have oncogenic properties when overexpressed in human neoplasias.

The aim of this study is to investigate gene expression levels of MAP2K1 and CCND1 in BDII rat model of endometrial adenocarcinoma cells. Quantitative real-time PCR was used to analyze expression levels of MAP2K1 and CCND1 genes in BDII/Han rat model of endometrial cancer cells using TaqMan approach. The differences in gene expression levels of MAP2K1 and CCND1 between pathologically EAC malignant and nonmalignant cells showed an upregulation of MAP2K1 and CCND1 in EAC malignant cells. The analyzed data presented observable mean differences between MAP2K1 and CCND1 in several endometrial cell lines that were examined.

Although no statistical significance was reached, an alteration in gene expression levels in malignant and nonmalignant endometrial cells could be observed. Furthermore, this present study shows observable upregulation of MAP2K1 and CCND1 in endometrial carcinoma cells vs. nonmalignant endometrium cells and encourages further investigation of the role of CCND1 and MAP2K genes in endometrial carcinogenesis.
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<th>Description</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>BN</td>
<td>Brown Norway</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1 gene</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycles to threshold</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>EC</td>
<td>Endometrial carcinoma</td>
</tr>
<tr>
<td>EAC</td>
<td>Endometrial adenocarcinoma</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>F1</td>
<td>First generation</td>
</tr>
<tr>
<td>F2</td>
<td>Second generation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAP2K/MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>N1</td>
<td>Backcross generation</td>
</tr>
<tr>
<td>NME</td>
<td>Nonmalignant endometrium</td>
</tr>
<tr>
<td>NUT</td>
<td>N1 progeny uterine tumors</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>REF</td>
<td>Rat embryo fibroblast</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
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1. Introduction

Endometrial carcinoma (EC) is the most frequent diagnosed gynecological type of cancer that develop in the inner lining of the uterus of the female reproductive tract. Endometrial carcinoma were diagnosed in more than 319,000 women in 2012 worldwide at the average age of 60 years (Cancer research UK, 2012). In Sweden, EC accounted for approximately 5% out of 27,688 malignancy cases in females in 2011 (National Board of Health and Welfare, 2013). There are two major subtypes of endometrial carcinoma, the type I that is depended upon estrogen and type II which is estrogen-independent, clinically more fast-growing and less common. The endometrial carcinomas of type I develop mostly in women in the premenopausal phase with an incidence rate of 80%, while type II EACs mostly arises in the postmenopausal phase with an approximate rate of 10-20%. Endometrioid adenocarcinoma is the most histologically common EC, whereas serous papillary carcinoma as well as clear cell carcinomas are referred to the more aggressive and poorly prognosed type II EC (Bokhman, 1983).

Studies and research on EC is difficult to conduct on human tumour samples due to the complex nature of tumor arousal and genetic heterogeneousness in the human population (Falck & Klinga-Levan, 2013). Therefore, inbred animal models can be a promising tool to use in EC research. BDII/Han inbred rat models have diligently been used since 1987 in order to conduct and advance endometrial adenocarcinoma research (Vollmer, 2003). Approximately 90% of the virgin female BDII/Han inbred rats induce carcinogenesis in a spontaneous manner throughout their lifetime (Deerberg & Kaspereit 1987). Additionally, BDII/Han rats have important similarities in histopathology and pathogenesis as humans and the carcinogenesis are hormonally dependant. These factors make BDII/Han inbred rat cells promising candidates to be used in study of endometrial adenocarcinoma (Samuelson et al., 2009).

1.1 Gene expression analysis using Reverse transcription polymerase reaction (RT-PCR)

RT-PCR is a technique used in medical research in order to synthesize DNA from an RNA template through reverse transcription. The mechanism of this method is to copy RNA into DNA molecules by reverse transcriptase enzyme and thereafter amplify the complementary DNA (cDNA) through classical PCR technique. This type of PCR method is a significant tool in gene expression analysis since mRNA of a cell is a representation of genes that are actively
being expressed (Muhlrad, 2003). For these reasons RT-PCR allows RNA to be studied with the same molecular approaches as in DNA investigations. Through this process, cDNA is achieved and can be used in polymerase chain reaction (PCR). The RT-PCR process is initiated by cDNA denaturation, which involve heating to 95°C. This eventually result in disruption of hydrogen bonds between complementary strands and thus yielding single stranded molecules (Cooper, 2007). The next step is to lower the temperature in order allow the complementary primers of the sequences to anneal. Thereafter, the temperature is once again increased to approximately 72 °C, which is the most favorable temperature for DNA polymerase activity (Chien et al., 1976). DNA polymerase will then synthesis DNA from the complementary strand. This process can be repeated many times which increase the DNA molecule amount per cycle. Thus, RT-qPCR can be used in order to analyze levels of gene expression in specific cells.

1.2 Real-time quantitative PCR

Information gained through gene expression processes is used to synthesize a functional gene product such as proteins, or non-protein coding genes (rRNA or tRNA genes) where the product is housekeeping and structural RNA. Quantitative PCR (qPCR) is a vital molecular biology research tool in cancer research and is the most powerful and sensitive technique to date. It is used in various applications such as gene expression analysis, drug target validation, discovery of biomarkers, detection of pathogens, genotyping and quantity RNA interference. Through the qPCR technique it is possible to determine expression changes of genes of interest by quantifying the abundance of the gene-specific transcript (Life technologies USA, 2010). These quantitative measurements of specific genes are essential towards the goal of understanding the fundamentals of cellular mechanisms and to detect alterations in gene expressions in response to e.g. growth factors or pharmacological agents in cancer (Bustin, 2000; Bustin 2002). Due to these properties, gene expression analysis using qPCR is significant tool to investigate gene expressions that are responsible for cancer development and determine response to different cancer treatments (Mocellin et al., 2003)

1.3 The Taqman approach

Real-time PCR is an effective, quick and easy in vitro method used in order to amplify a specific DNA fragment using DNA polymerases. One of the DNA polymerases that is commonly used is Taq DNA polymerase which is isolated from Thermus aquaticus bacteria. Taq DNA polymerases are able to synthesize DNA strands from a template with
deoxyribonucleotide triphosphates (dNTPs), magnesium ions, primers and proper buffer systems (Old & Primrose, 1994; Valasek & Repa, 2005). The polymerase chain reaction consist of two oligonucleotide primers which are located on each side of the target DNA sequence that has to be amplified. After the denaturating process, the primers in the reaction cross to opposite sides of the DNA and allows the polymerase enzyme to synthesize complimentary DNA between the two primers (Old & Primrose, 1994). Taqman probes consist of a fluorescent reporter dye which is located at its 5’ end and a quencher dye at the 3’ end (Wong & Medrano, 2005; VanGuilder et al., 2008). When the Taqman probes are close to one another, in a unbound state, the quencher reduces the reporters fluorescence intensity by fluorescence resonance energy transfer (FRET) which result in no detection of reporter fluorescence (Bustin, 2000). Alternatively, when the PCR product is generated, the probe can bind to the target sequence and remain hybridized till the enzyme reaches the probe. During the extension step of the PCR process, the 5’ to 3’ exonuclease activity of DNA polymerase enzyme degrade the probe. The 5’ reporter dye is released from the quenching effect of the 3’ dye due to polymerase enzyme 5’ activity leading to detectable increase in fluorescence intensity (Heid et al., 1996).

1.4 Regulation of cyclin D1 expression and cell cycle progression by mitogen-activated protein kinase cascade

There are various signaling pathways which involve multiple regulatory molecules that are associated with carcinogenesis of endometrial carcinoma. One of the signaling pathways that has been identified to be associated with development of endometrial carcinoma is the mitogen-activated protein kinase pathway (MAPK) (Ma et al., 2014). MAPK pathway is responsible for differentiation and proliferation of cells through RAS-RAF-MEK-ERK signaling cascade. In cancer, activation of the MAPK pathway is frequently stimulated when growth factors and their associated receptors are overexpressed (Hoshino et al., 1999). Upon the binding of growth factors to their receptors, the RAS-RAF-MAP2K signaling cascade serves to shift the growing stimuli from RAS proteins through MAP2K1/2 to MAPK1/3 kinase or the extracellularly located MAPK3/1 kinase (Kolch, 2000; Peyssonnaux & Eychene 2001). After the MAPK3/1 proteins are phosphorylated they are translocated to the nucleus where they activate transcription factor ERK1 to serve in the cell proliferation process (Figure 1) (Pages et al., 1993). Activation of MAPK3/1 also stimulate cyclin expression which posses important roles in the cell cycle (Sherr 1995; Nurse 1997). Progression through the cell cycle requires expression of cyclin proteins, which form complexes with cyclin-dependent kinases.
(CDKs). This eventually result in production of holoenzymes that phosphorylates proteins that are needed for progression through the cell cycle. Cyclin D1 which is a proto-oncogene that binds to CDK4 or CDK6 and induce a rate limiting event through the G1 phase in the cell cycle’s progression into S-phase (Klein & Assoian, 2008) Levels of cyclin D1 is increased by mitogenic growth factors during the G1 phase through the MAPK pathway which increases transcription frequency of cyclin D1 and also inhibits proteolysis and export of cyclin D1 from the cell’s nucleus (Alao, 2007; Böhmer et al., 1996). In the G1 phase, cyclin D1 is synthesized and subsequently accumulated in the nucleus of the cell and is degraded while the cell enters the S phase (Baldin et al., 1993). More specifically, when cyclin D1 forms complex with CDK4 it induces progress through the G1 phase by phosphorylation, inhibition, of retinoblastoma protein (RB). Inhibition of retinoblastoma protein promotes the G1/S phase transition and DNA synthesis by transcription factor E2F which transcript genes that are necessary for S phase entry (Figure 2).

Additionally, cyclin D1 also functions independently of CDKs by binding to receptors, e.g. thyroid hormone receptors, estrogen receptors and peroxisome proliferator-activated receptors.

Figure 1. MAPK signaling pathway. RAS GTPase activation induce activity of the RAF protein kinase. The activated RAF kinase subsequently phosphorylates and thus activates MEK1/2 and ERK1/2 kinases respectively. The phosphorylated and activated ERK1/2 proteins shift their location to the nucleus where they induce stimulation of transcription factors. This cascade eventually leads to increased CCND1 gene expression, which promotes proliferation and survival of the cell (Adapted from Gowrishankar et al., 2013).
that are needed for normal cell proliferation and differentiation or by binding to histone acetylase and histone deacetylases in order to regulate genes for the proliferation process in the G1 phase (Wang et al., 2004; Fu et al., 2004).

1.5 Objectives

The aim of this study is to culture BDII/Han rat EC cells, determine the cells proliferation by conducting MTS assay and to investigate gene expression levels of MAP2K1 and CCND1 through quantitative real-time PCR analysis. The qPCR analysis will be able to provide information whether CCND1 and MAP2K1 are overexpressed in pathologically malignant BDII rat cells in comparison to nonmalignant cells. Cell culturing will contribute to maintaining cells and using them for the qPCR and MTS analysis. The MTS assay will determine the quantity of proliferating viable cells and microscopy will be able to reveal how aggressively the cultured cells proliferate and reach an approximate confluence of 80%. These analyzes have the capability to give an insight to CCND1’s and MAP2K1’s involvement in endometrial carcinogenesis and confirm the examined cells pathology and proliferation.

Figure 2. Schematic illustration demonstrating the regulation of the cell cycle and the G1/S phase transition through cyclin D1, CDK4/6 and p16. Extracellular signals increase cyclin D1 levels which in turn phosphorylates retinoblastoma protein (RB). Phosphorylated RB induce cell cycle progression and DNA synthesis through E2F transcription factor. Alternatively, p16 inhibits CDK4/CDK6 and thereby prevents G1-to-S phase progression (Adapted from Peurala et al., 2013).
2. Materials and methods

2.1 Tumor material achieved through BDII/Han rat crosses

The endometrial BDII rat tumor cells that were used in this study was obtained from a previous study conducted by Karlsson et al., 2009. The tumor material was received by crossing BDII/Han females with SPRDCu3/Han and BN/Han males which provided a F1 progeny. Thereafter, males and females from F1 progeny were crossed in order to produce a F2 offspring. Subsequently, N1 were produced by backcrossing the F1 males to BDII females. The female rats of the N1 progeny were carefully examined for endometrial tumors by palpation each week. When these examinations lead to suspicion of tumor development, the female rats were euthanized and the uterine tumor was surgically removed. This tumor tissue was later used to constitute cell cultures. Moreover, pathological analysis were performed on the tumor tissue and suggested that a greater part of the N1 progeny tumors were endometrial adenocarcinoma, whereas a smaller portion of the surgically removed cellular mass showed no malignant endometrium cells (NME). The cell lines used in this present study are N1 progeny uterine tumors (NUT) that are both classified as endometrial adenocarcinoma and nonmalignant cells (Karlsson et al., 2009). Totally 14 cell lines of the NUT were studied of which 8 are classified as malignant cells and 6 nonmalignant cells. Additionally, GAPDH housekeeping gene were used as endogenous control, whereas Rat embryo fibroblast cell line (REF) was used as exogenous control respectively.

Figure 3. Schematic illustration of BDII and SPRDCu3/BN rat crosses. Males with low incidence rate of EAC development (SPRDCu3 or BN) were crossed with female EAC susceptible BDII rats which develop spontaneous tumors in 90% of the time during their lifespan. The initial rat cross provided an F1 progeny which in turn was backcrossed to BDII female rats that provided N1 progeny.
2.2 Cell culturing of N1 progeny uterine tumor cells

The cell lines NUT50, NUT97, NUT110 and NUT129 were cultured in Dulbecco’s modified Eagle medium (DMEM) with addition of 5 ml MEM vitamin solution (100x), 10 ml MEM amino acids (50x) solution, 5 ml MEM non-essential amino acid solution (100x), 5 ml L-glutamine solution, 5 ml penicillin-streptomycin (PEST) and 50 ml fetal bovine serum (FBS) for malignant NUT cells and 100 ml for non-malignant cells. The cultured cells were incubated and grown in T75 flasks at 37°C with 95% humidity and 5% CO₂. After incubation, the cells were inspected under microscope with x10 magnification and harvested through trypzination at 80-90% cell confluence.

2.3 Isolation of RNA, cDNA synthesis and qPCR

The total RNA from aforementioned NUT cells was isolated according to manufacturer’s protocol in mirVana™ miRNA isolation Kit (Ambion). Thereafter, both the quality and quantity of the RNA samples were established by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) where all the samples were within 260/280 absorbance ratio of 0.69-2.14 (Table 2). Calculations were made for the cDNA synthesis with 1000 ng of total RNA per 20 μL reaction for all the NUT samples according to High Capacity RNA-to-cDNA kit protocol (Applied Biosystems). All reagents in the High Capacity RNA-to-cDNA kit were added into fresh PCR tubes, specifically; 10 μl of RT buffer 2X, 1 μl of Enzyme mix 20X, accordingly calculate RNA volume and nuclease-free water up to a total volume of 20 μl. The PCR tubes were incubated in 37 °C for 60 minutes. Subsequently, the reaction was stopped by heating to 95°C for 5 minutes and holding at 4°C in the thermal cycler. After the thermal cycler finished, a total of 10 μl of cDNA were diluted in 90 μl of nuclease-free water in fresh eppendorf tubes for every sample. After this procedure the cDNA was ready to be used in real-time PCR application. A reaction mix was prepared for the 96-well plate according to table 1.

Table 1. Reaction mix for 96-well plate

<table>
<thead>
<tr>
<th>Reaction mix x1</th>
<th>Reaction mix x100 (2 separate eppendorf tubes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>Master mix</td>
<td>5 μl</td>
</tr>
<tr>
<td>Taqman probe</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Template (cDNA)</td>
<td>2 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Quantity of reagents used for quantitative real-time PCR
Real-time qPCR was performed on the cDNA from the endometrial cells with TaqMan Universal PCR Master Mix (Life Technologies USA, 2010) in a PikoReal real-time PCR system (Thermo Scientific, MA) using the TaqMan probes for MAPK and CCND1 respectively (Life technologies USA, 2010). Real-time PCR cycles consisted of: 5 minute at 25°C, 30 minutes at 42°C and 5 minutes at 85°C. The Cq values of each sample was normalized to the reference gene GAPDH.

2.4 Analysis of data

For the data analysis, comparative threshold method was used in order to calculate relative quantities for MAP2K1 and CCND1. The fold change were calculated accordingly to $2^{\Delta\Delta Ct}$ (sample – ΔCt calibrator) where GAPDH is the endogenous control and REF is the calibrator. The calibrator and GAPDH ΔCt values were obtained by subtraction of target gene’s from the GAPDH values.

The comparative threshold value provide information about the cycle number at which the fluorescence passes the defined threshold (Life technologies USA, 2010). Gene expression differences in NUT cells were determined by Student’s t-test (p <0.05). One-way analysis of variance (ANOVA) were used to test statistical significance among the triplicates (p <0.05). The data analysis were performed in GenEX software (MultiD Analyses AB, Göteborg, Sweden).

2.5 Cell count and MTS assay

MTS assay was performed on three out of the four cell lines that were cultured, namely NUT50, NUT97 and NUT110 respectively. The incubated T75 flasks with each cell line were trypzinated when they had reached approximately 80% confluence. A total of 40 μl of the cell suspension was added to an 1.5 ml eppendorf tube, which subsequently was supplemented with 40 μl of trypan blue and a total of 10 μl of the mixture was pipetted into a Bürker hemocytometer. All the viable cells were counted under microscope at x10 magnification in A-squares consisting of 1 mm². For every cell line, a total of 100 μl (7700 cells) were seeded into 8 wells in four 96-well microtiter plates which will represent 24h, 48h, 72h and 96h respectively. After each timeframe, one 96-well microtiter plate for every cell line were supplemented with 20 μl of CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) and incubated for one hour. The plates were later analyzed in FLUOstar Omega.
96-well microplate reader recording the absorbance at 490 nm in Omega data analysis software (BMG Labtech).

3. Results

3.1 RNA quality and concentration measurement with NanoDrop

The purity and concentration of the RNA were determined for both endometrial adenocarcinoma cells (EAC) and nonmalignant cells (NME) by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) at \(A_{260}/A_{280}\) nm and \(A_{260}/A_{230}\) nm respectively (Table 2). The RNA in the samples contribute to the total absorbance of the samples since RNA absorb at 260 nm and a ratio of ~2.0 is commonly considered “pure” for RNA measurements. If absorbance ratios after measurement is significantly below ~2.0, it might be an indication of contaminants, phenols or proteins being present in the sample that absorb at 280 nm. Moreover, \(A_{260}/A_{230}\) ratio usually have higher “pure” ratios in comparison to \(A_{260}/A_{280}\) nm with an expected value of 2.0-2.2 as consideration to “pure” (Thermo Scientific, 2008).

Table 2. Quality and quantity of RNA determined by NanoDrop ND-1000

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pathology</th>
<th>RNA Conc. (ng/μl)</th>
<th>Abs. (260/280)nm</th>
<th>Abs. (260/230)nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUT50</td>
<td>EAC</td>
<td>138.3</td>
<td>1.98</td>
<td>2.17</td>
</tr>
<tr>
<td>NUT47</td>
<td>EAC</td>
<td>123.0</td>
<td>2.12</td>
<td>1.38*</td>
</tr>
<tr>
<td>NUT98</td>
<td>EAC</td>
<td>131.8</td>
<td>0.69*</td>
<td>0.61*</td>
</tr>
<tr>
<td>NUT128</td>
<td>EAC</td>
<td>536.0</td>
<td>2.11</td>
<td>2.10</td>
</tr>
<tr>
<td>NUT41</td>
<td>EAC</td>
<td>375.6</td>
<td>2.13</td>
<td>2.12</td>
</tr>
<tr>
<td>NUT42</td>
<td>EAC</td>
<td>111.0</td>
<td>1.98</td>
<td>1.87</td>
</tr>
<tr>
<td>NUT97</td>
<td>EAC</td>
<td>276.2</td>
<td>2.23</td>
<td>1.85</td>
</tr>
<tr>
<td>NUT46</td>
<td>EAC</td>
<td>335.7</td>
<td>2.12</td>
<td>1.98</td>
</tr>
<tr>
<td>NUT89</td>
<td>NME</td>
<td>635.3</td>
<td>2.10</td>
<td>2.08</td>
</tr>
<tr>
<td>NUT110</td>
<td>NME</td>
<td>407.1</td>
<td>2.14</td>
<td>1.92</td>
</tr>
<tr>
<td>NUT129</td>
<td>NME</td>
<td>335.0</td>
<td>2.12</td>
<td>1.91</td>
</tr>
<tr>
<td>NUT48</td>
<td>NME</td>
<td>226.3</td>
<td>2.12</td>
<td>1.83</td>
</tr>
<tr>
<td>NUT56</td>
<td>NME</td>
<td>151.0</td>
<td>2.05</td>
<td>1.98</td>
</tr>
<tr>
<td>NUT75</td>
<td>NME</td>
<td>102.2</td>
<td>2.13</td>
<td>2.10</td>
</tr>
<tr>
<td>REF</td>
<td>-</td>
<td>234.9</td>
<td>2.09</td>
<td>1.65*</td>
</tr>
</tbody>
</table>

* Appreciably lower ratio values in contrast to be considered “pure”
3.2 Expression levels of MAP2K and CCND1 in EAC and NME cells

Differences between pathologically malignant and nonmalignant endometrial cells (EAC vs NME) were tested by Student’s t-test for both MAP2K1 and CCND1 (Figure 4). The null hypothesis assumed no statistical difference in expression levels between malignant and nonmalignant cells with a 95% confidence interval ($\alpha = 0.05$). There is an observable difference of MAP2K and CCND1 gene expression between EAC and NME cells. The t-test results however, did not present a statistical significant difference in gene expression between endometrial adenocarcinoma tumor cells and nonmalignant cells with a p-value of 0.22 for MAP2K and 0.10 for CCND1 respectively.

![Figure 4. Expression levels of MAP2K and CCND1 in endometrial adenocarcinoma tumor cells and nonmalignant cells.](image)

The qPCR procedure measures the expression of the genes from a sample in every well. This specific measurement is shown as Cycles to Threshold (Ct), a value that is relative and which represents the amount of RNA that reach the threshold level. Generally, every cycle in the PCR process contributes to approximately a duplication of RNA. The Ct value is in logarithmic scale and are conversely proportional to the amount of RNA. Hence due to these factors, high $\Delta$Ct values represent low expression of the gene whereas a low $\Delta$Ct value
represents highly expressed genes. The fold change can be calculated accordingly by comparing the normalized expression (ΔCt). The fold change is a representation of the actual expression ratio where a positive fold change indicates that the gene of interest is upregulated while a negative fold change value indicates a downregulation of the gene (Livak and Schmittgen 2001). The fold change values were calculated for MAP2K and CCND1 in both malignant and nonmalignant BDII/Han rat cells. Both MAP2K and CCND1 had a positive fold change and expressed 1.96 and 1.62-fold increase respectively in comparison to nonmalignant BDII/Han rat cells.

3.3 Comparison of MAP2K and CCND1 expressions in all examined cell lines

One-way analysis of variance (ANOVA) was conducted in order to determine if there was a statistical significance between MAP2K and CCND1 among all examined cell lines. ANOVA test is a method which enables simultaneous comparisons between means and yields mean values that can be used in order to determine whether there is a significant relation between variables (Bewick et al., 2004). As with the Student t-test, the null hypothesis assumed no statistical difference in expression levels between MAP2K and CCND1 with a 95% confidence interval (α = 0.05). However, there is noticeable downregulation in log2 expression of MAP2K in NUT98 (-0.125) and NUT56 (-1.125) in comparison to CCND1 expression of 2.8 and 2.0 (Figure 5). Moreover, the result demonstrated that CCND1 was more expressed than MAP2K in all examined cell lines except NUT47 with a log2 expression of 1.5 (Figure 5). In order to analyze a correlation (if any) among the expression of MAP2K1 and CCND1 genes, Pearson’s correlation was tested. However, no significant correlation between the genes were observed.
3.4 Cell viability analysis by MTS assay

The MTS cell proliferation assay is a spectrophotometric technique used to determine quantity of proliferating viable cells. The MTS reagent includes tetrazolium (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) which is bioreduced by viable cells into medium-soluble chromogenic product formazan which acts as a light absorbent. The bioreduction process is performed by NADPH or NADH which is produced by dehydrogenase enzymes during metabolic activity of cells (Berridge & Tan 1993). The maximum absorbance for formazan is reached at 490 nm wavelength. Thus, the amount of formazan measured at 490 nm directly reflect the quantity of viable cells in the cell culture medium (Promega, 2012). The plotted results obtained from the MTS absorbance measurement show an increase in proliferation among all three cell lines from 24h to 96h (Figure 6). Both pathologically malignant NUT97 and NUT50 demonstrated a stable proliferation from 24h to 48h where they later increased and reached their proliferation peak at 72h. However, at 96h their proliferation decrease in comparison to the 72h absorbance measurement. The nonmalignant NUT110 results demonstrated a decrease in formazan absorbance from 24h to 48h and later continued to increase at 96h.
4. Discussion

Majority of studies performed on MAP2K and CCND1 has shown that their altered expression play a crucial role in carcinogenesis. CCND1 has been demonstrated to have oncogenic properties when overexpressed in human neoplasias such as pancreatic cancer (Biliran et al., 2005; Chung et al., 2000), breast cancer (Hosokawa et al., 1998; Velasco-Velázquez M et al., 2011) and endometrial carcinoma (Quddus et al., 2002; Soslow et al., 2000). Similarly, overexpression of MAP2K in different types of cancer have been demonstrated in numerous studies such as liver cancer (Huynh et al., 2003) and colorectal cancer (Fang and Richardson 2005). The MAPK pathway play a critical role in cells survival, proliferation and differentiation. The pathway is responsible for transducing extracellular ligand signals of the receptor tyrosine kinases to transcription factors in the cell which regulate gene transcription in the nucleus (Yang et al., 2003). Therefore, mutations in the MAPK pathway can be detected in many types of cancers, which result in hyperactivity of pathway signaling and uncontrolled cellular growth. Combining gene expression profiling
with statistical methods are essential tools towards the goal of analyzing and understanding complexity of carcinogenesis (Karlsson et al., 2009). One of the most essential methods for gene expression analysis is quantitative polymerase chain reaction (qPCR) due to its high sensitivity in small gene expression changes and its high specificity. There are several factors that affect the qPCR expression values, quality and amount of the cells examined, yields of the extraction and the efficiency of the reaction (Jurcevic et al., 2013). In order to approach these factors, it is of high importance to use normalization strategies that are suitable for the data analysis. One strategy that is commonly used for normalization is endogenous control genes. In order for endogenous control genes to be suitable they must have stable expression among all samples (Kubista et al., 2009; Huggett et al., 2005).

In this present study, expression levels of MAPK2K1 and CCND1 were compared between endometrial adenocarcinoma tumor cell lines and nonmalignant endometrial samples from BDII rats. In total, 14 specimens were examined out of which 8 cell lines had their origin from endometrial adenocarcinoma tumors and 6 from nonmalignant lesions. CCND1 and MAP2K had differential gene expression in comparison to nonmalignant cells. Although the student t-test did not provide statistical significance for MAP2K and CCND1 gene expression in malignant and nonmalignant cells an indication of upregulation can be observed in Figure 4. The relative log 2 expression of MAP2K and CCND1 in endometrial malignant and nonmalignant cells (Figure 5), show a downregulation of MAP2K in cell lines NUT98 (malignant) and NUT56 (nonmalignant). There is a possibility that the RNA obtained in these aforementioned cell lines were contaminated due to their low A_{260}/A_{280} and A_{260}/A_{230} values that are not within the “pure-interval”. This could have contributed to the ANOVA result observed in NUT98 and NUT56 respectively.

Additionally, in nonmalignant cells, cyclin D1 has the capability to sense the microenvironment before the cell enters the cell cycle due to that extracellular signals are required in order to induce the cell cycle progress. This type of cellular control can be disrupted in different types of cancer which leads to overexpression of cyclin D1. Since cyclin D1 promotes the cell cycle progress, specifically G1/S phase transition, an uncontrollable cellular differentiation and proliferation emerges. Moreover, the mitogen-activated protein kinase pathway (MAPK pathway) is correlated with cyclin D1 expression since mitogenic growth factors are one of the cyclin D1 gene transcription activators (Klein & Assoian, 2008). The MAPK pathway holds an extensive part in mitogenic signaling. Through the RAS-RAF-MEK-ERK signaling pathway AP-1 transcription factors, Fos and Jun can be stimulated for
increased expression. The promoter of cyclin D1 consists of a AP-1 site, which is appropriately regulated by both Fos and Jun (Klein & Assoian, 2008). In this manner, when there is an increased activity of the mitogen-activated protein kinase pathway an increase in cyclin D1 expression can be observed (Figure 5).

A statistical significant overexpression of both CCND1 and MAP2K was probably not reached due to the small sample size. Since the confidence interval used in ANOVA and Student’s t-test were 95%, it is highly unlikely that the null hypothesis is inaccurate. Therefore, the results obtained from this study encourages continuous investigation of the transcriptional regulation of CCND1 by MAP2K in endometrial carcinoma due to observable upregulation of MAP2K and CCND1 in malignant EAC compared to nonmalignant cells (Figure 4). Additional important factor is that BDII/Han rats have important similarities in histopathology and pathogenesis as humans that can be exploited to design future research interventions to study the role of genetic regulation in endometrial carcinogenesis (Samuelson et al., 2009).

Culturing cells in vitro have a tendency to follow a typical growth pattern. Lag-phase is the first phase the cells enter after they have been cultured. In this phase the cells are adapting to the culture environment and proliferate in slower rate. After the cells have adapted to the culture environment, they enter the log-phase (logarithmic phase) where they start to consume the nutrients in the culture medium and proliferate exponentially. After all of the nutrients in the culture medium have been spent, the cells reach a stationary phase resulting in decreased proliferation (Thermo Scientific, 2016). The MTS results obtained in this study demonstrate the lag-phase for all three cell lines (NUT50, NUT97 and NUT110) at 24h to 48h. During the 72h point measurement all three cell lines had reached their exponential phase and thereby started consuming the nutrients in the cell culture medium. Subsequently during the 96h mark, the malignant cell lines NUT50 and NUT97 reached their stationary phase and a decrease in proliferation could be observed. However, the nonmalignant NUT110 were still proliferating and in exponential phase after 96h of incubation (Figure 6). The overall MTS results indicate that the malignant NUT97 cell line has a very aggressive proliferating tendency in comparison to NUT50 and NUT110. These results were expected due to that NUT97 reached 80% confluence within shorter incubation period than the other cell lines during the cell culturing process. However, there is a decline in proliferation for NUT97 at 96h, probably due to decreased amount of nutrients in the cell culture medium since higher quantity of cells are present and the MTS assay only takes viable cells into consideration. Most tumor cells
consume nutrients at significantly higher rate in comparison to normal cells. This eventually leads to cell death in the cell culture due to an inadequate supply of nutrients (Medina et al., 1992). The pathologically malignant NUT50 has the same proliferating pattern as NUT97, i.e. it grows and proliferates during 24h-72h where it later declines at 96h. The nonmalignant NUT110 showed an increase in proliferation through 24h to 96h, probably due to greater amount of fetal bovine serum in the cell culture medium in comparison to the malignant cell lines. The MTS assay indicated that NUT50 proliferated at a higher rate in comparison to NUT97 and NUT110 within the first 72h. These MTS results may have a connection to the qPCR results which demonstrated highest expression levels of MAP2K and CCND1 in NUT50. However, the NUT110 cell line had similar expression levels of CCND1 and MAP2K as NUT97. This could also be observed in the MTS results where both NUT110 and NUT97 had similar proliferation pattern. These observations strongly suggests that expression levels of MAP2K and CCND1 are associated with proliferation of NUT cells.

This present study encourages further research and qPCR analysis of MAP2K and CCND1 due to the statistical insignificant results obtained from ANOVA and the Student’s t-test. There is a possibility that a statistical significant result could be achieved if a similar study was conducted with greater sample size and performed under the same conditions. The continuos research on BDII rat tumor cells can lead to better understanding of MAP2K’s and CCND1’s molecular mechanisms and crucial role in carcinogenesis. This may eventually lead to new possible therapeutic treatments not only for endometrial carcinoma but also for many various cancer types.

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6. References


