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Extracellular Matrix (ECM) and Cell Adhesion Molecules (CAMs) gene expression in brain GD-10, Hippocampal cell (mHT-22 Cell Line), human glioblastoma-astrocytoma (hLN-405), and rat glioma cell (rF98) using real time PCR

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GRAPHICAL ABSTRACT

ABSTRACT

The aim of this study is to compare ECM and CAMs gene expression in hippocampal cell, glioblastoma-astrocytoma and glioma cell using real time PCR at gestation day of 10 (GD-10). Result of real time PCR showed that expression level of extracelluler matrix of vimentin was higher than the expression of fibronectin, Neural cell Adhesion molecule (Ncam), neurofilament high (Nfh), neurofilament medium (NFm), neurofilament low (Nfl) and tenascin. The expression of vimentin in the brain also shows the highest when compare to the expression in the human glioblastoma-astrocytoma (hLN-405), rat glioma cell (rF98) and mouse hippocampal cell line (mHT-22). The number of vimentin gene copy was 538.554, whereas in the culture of hLN-405 was 2.246.309, in culture of rF98 was 974.368 and mHT-22 was 1.542.529. These findings suggested that vimentin most dominant expressed compare to the another gen. Expression of vimentin was gradually lower from astrocyte cell (hLN-405 or human glioblastoma-astrocytoma), then in hippocampus cell, glioma cell and embryonic brain cell.

Keywords: vimentin, brain, Hippocampal Cell, glioblastoma-astrocytoma, glioma cell, brain E-10

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1. INTRODUCTION

Adhesive interaction between neurons and extracellular matrix play a key role in neuronal pattern formation. Molecules of the extracellular matrix have been implicated to play a pivotal role in tissue morphogenesis [1]. In addition, extracellular matrix and cell adhesion also plays a role in the process of brain morphogenesis. Cell adhesion systems should be regarded as molecular machineries that translate basic genetic information into complex three-dimensional patterns of cells in tissues [2]. Assembly of the central nervous system (CNS) architecture during development and maintenance of its circuitry throughout life are largely dependent on cell adhesion molecules's (CAMs) capability of stabilizing and modulating cellular interactions. The neural cell adhesion molecule (NCAM) is a well characterized cell adhesion molecule. It is implicated in various morphogenetic processes during development, such as proliferation, migration, differentiation, and synapse formation [3].

In the process of development of the nervous system or brain, vertebrate evolves in a well-defined temporal sequence of events which includes proliferation of epithelial cells stem migration of neuronal precursors from ventricular zone to target area in neural tube. Neuron-glia cells interactions play a crucial role in several of these processes. In this context, cell adhesion molecules (CAMs) and the extracellular matrix (ECM) are involved in the control of neuronal migration and axon elongation [4]. The extracellular matrix (ECM) of the embryonic brain is composed of many types of molecules that have distinct patterns of spatial and temporal expression. Many of these components were originally discovered in non-neural tissues include fibronectin (FN), tenascin (TNC), Neurofilament, Ncam, Vimentin.

Fibronectin is extracellular matrix believed to be involved in many cellular functions such as cell adhesion, wound healing and cell migration [5]. These proteins are present in the central nervous system and are considered to play important roles in the development of embryonic neurons and regeneration of damaged adult nerves fibers.

Tenascin is extracellular matrix molecule too, synthesized and released by young astrocytes during embryonic and early postnatal development of the nervous system. Distinct spatial and temporal distributions of tenascin during developmental events suggest a role in neuronal guidance.

The neural cell adhesion molecule (Ncam) is the one of most studied and well characterized cell adhesion molecules. It is implicated in various morphogenetic processes during development, such as proliferation, migration, differentiation, and synapse formation [3].

Vimentin is a protein particulary useful as markers of glial differentiation [6]. Vimentin is an intermediate filament component of astroglial cells. The Function of vimentin in the cells is supposed to play role in neurogenesis because of its co expression with neurofilament in neuroblast. More recently, evidence has been published on the role of vimentin in DNA replication and recombination, DNA repair and in gene expression.

Three sub-units of Neurofilament (NFs) are expressed at distinct stages of vertebrate development, triggered by neuron differentiation. Neurofilaments are intermediate filaments of neurons that are considered to add rigidity, tensile strength and possibly intracellular transport guidance to axons and dendrites. Exclusively expressed in neurons, NFs are members of the cytoskeleton proteins that act together to form and maintain cell shape and facilitate the transport of particles and organelles within the cytoplasm [7].

This study will determine the difference of extracelluar matrix (ECM) and Cell Adhesion Molecules (CAM) gene expression on brain at GD-10, hLN-405 (human, glioblastoma-astrocytoma), rF98 (rat glioma cell) and mHT-22 (mouse hippocampal cell line) at the levels of mRNA. Primers of GAPDH, Fibronectin, Ncam, Tenascin, Vimentin, Neurofilament heavy, medium and low are used in this study, was designed by *Biotez Berlin-Buch GmbH, Berlin* ,Germany.

2. EXPERIMENTAL

2.1 Experimental Animals and Sample Collection

Black-6 mice were used as experimental, from Charite Universitats Medizin Berlin, Jerman. Rearing the animals was done in a room at 23-27°C and 83% humidity. Food and water were given ad libitum. When female mice achieved their sexual maturity (10-12 weeks old), they were mated with a male (1:1). A vagina plug detected the following morning was defined as day 0 of gestation day [8]. Cells hLN-405 (human, glioblastoma-astrocytoma), cells rF98 (rat glioma cell) and cells mHT-22 (mouse hippocampal cell line) from SIGMA were used in this experiment.

The balck-6 mice pregnant were killed by cervical dislocation at GD-10. The pregnant mice were cut opened, next uterus was taken and put in falcon tube containing buffer solution. Uterus were opened, embryo was taken and brain were isolated, under stereo microscope. Brains sample were put in Nunc cryo tube, and kept in box which containing liquid nitrogen. Part of the entire brain sample was put in the tube, which containing RNA-*later* (Sigma), for analysis DNA.

2.1.1 Reverse Transcriptase and Real Time RT-PCR

The total RNA brain tissue was extracted with the RNeasy kit according to the manufacture's protocols. cDNA was synthesized from the total RNA using the *Qiagen One Step RT-PCR Kit* (Cat. No.210210). PCR reactions using enzymes *AidTM H Minus M-MuLV RT* (Cat. No. 130 125 486) at a temperature of 95°C, 7 min, 45 cycles of PCR (20 sec, 95ºC, 60°C, 20 sec,72°C, 30 sec), 42°C, during 1 hour 15 minutes, 70°C elongation then followed with the temperature of 70°C, for 5 minutes. Quantitative analysis performed by Real-Time PCR. Analysis of Polymerase chain reaction (PCR) is done by adding each cDNA 9 µl of control brain and 1 µl of Primary-Mix into each different tube. In our experiments, *Primary-Mix* consists of eight primary types of GAPDH, Fibronectin1, vimentin, tenascin, Ncam1, NFh, Nfm, and the Nfl. Brain tissue of embrio, and then was added by the component of reactions from SYBER Green kit Qiagen. Then reaction of Real Time RT-PCR showed the complete series of targetted cDNA, followed by Oligonucleotide primers. The Primers used in this study were synthesized into *Biotez Berlin-Buch GmbH, Berlin*, Germany (table 1).

Table 1 Sequencing of primer position (f = forward; r = reverse), % G/C

Primer	Sequence (5´ ⟶ 3`)	GC (%)	TIB reference no.
GAPDH f	CCA TCA CCA TCT TCC Agg AgC gA	56,5	017079:553U23
GAPDH r	ggA TgA CCT TgC CCA CAg CCT Tg	60,9	17079:977L23
Fibronectin-f	AGG CAT AAG GTT CGG GAA GAG GT	52,2	005403 69362F23
Fibronectin –r	GCA GTT GTC ACA GCG CCA GCC	66,7	005403 77639R21
NCAM-f	GGT GCA GTT TGA GCC AGA GG	56,5	001081445:1891F23
NCAM-r	CGT CCT CTC CCA TCT GCC CTT C	63,6	001081445:2162R22
Tenascin-f	CTA CAG CCT GGC AGA CCT GAG	61,9	011607.2:5397F21
Tenascin-r	CTT GTA GGT CCA CCC GGA GCT	61,9	011607.2:5792R21
Vimentin-f	CTG AGG CTG CCA ACC GGA ACA A	59,1	011701.3:1376F22
Vimentin-r	CCT CGC CTT CCA GCA GCT TCC	66,7	011701.3:1682R21
Nf h-f	AGG AGA TAA CTG AGT ACC GGC G	54,5	010904.3:1071F22
Nf h-r	CCA AAG CCA ATC CGA CAC TCT TC	52,2	010904.3:1349R23
Nf m-f	GTG GTT CAA ATG CCG CTA CGC C	59,1	008691.2:1055F22
Nf m-r	GAG GCC CGG TGA TGC TTC CTG	66,,7	008691.2:1432R21
Nf l-f	TGG CCT TGG ACA TCG AGA TTG CA	52,2	010910:1221F23
Nf l-r	GCT TCT CCT TCA GAG GGG GGC	66,7	010910:1489R21

2.1.2 DNA Electrophoresis

The separation of DNA in the agarose gel was performed according to base pair DNA, followed by separation marker 1kbp. Each of 1 µl sample brain GD-10, hLN-405 (human, glioblastoma-astrocytoma), rF98 (rat glioma cell) and mHT-22 (mouse hippocampal cell line) are pippeted to well agarose gel. Before running at 80 V, agarose solution was added 5µl Ethidiumbromide. Gel agarose was transferred on to box photo UV, to see the bands of DNA.

3. RESULTS & DISCUSSION

These results show the expression of some gene in the brain of mice fetuses at GD-10, including hLN-405 (human, glioblastoma-astrocytoma), rF98 (rat glioma cell) and mouse hippocampal cell line (mHT-22). The results are shown in Figure 1, 2, 3 and 4. The expression of electrophoresis of cDNA fibronectin (FN1), Ncam 1, tenascin (Tnc), vimentin (Vim), Neurofilament high (Nfh),

Neurofilament medium (Nfm) and Neurofilament low (Nfl) are shown at 462bp, 416 bp, 327bp, 301bp, 398bp and 289bp. Fibronectin and Nfh were very low expressed compare to the other genes (figure 1, 2, 3, dan 4). Nfm dan Nfl weren't expressed (Figure 2, 3 and 4).

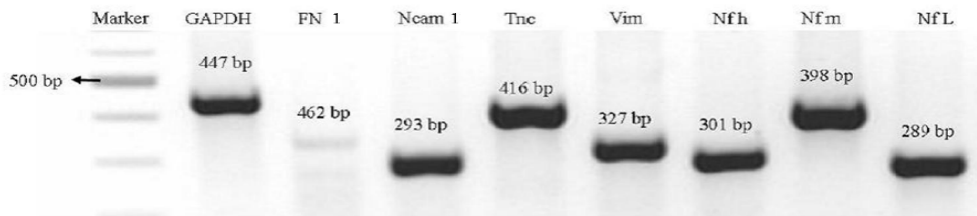

Fig. 1 Expression gene of brain embryo at gestation days (GD-10) by DNA electroforesis. Marker, GAPDH, FN: Fibronectin, Ncam 1, Tnc: Tenascin, Vim: Vimentin, Nf h: Neurofilament high, Nf m: Neurofilament medium, Nfl: Neurofilament low.

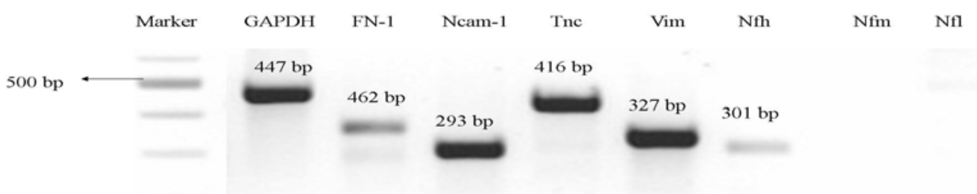

Fig. 2 Gene Expression of mHT-22 Cell Line by DNA electroforesis. Marker, GAPDH, FN: Fibronectin, Ncam 1, Tnc: Tenascin, Vim: Vimentin, Nf h: Neurofilament high, Nf m: Neurofilament medium, Nfl: Neurofilament low.

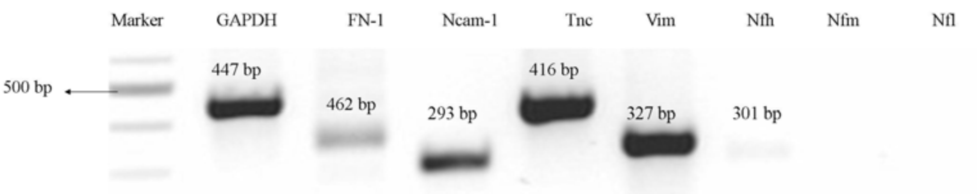

Fig. 3 Gene Expression of hLN-405 by DNA electroforesis. Marker, GAPDH, FN: Fibronectin, Ncam 1, Tnc: Tenascin, Vim: Vimentin, Nf h: Neurofilament high, Nf m: Neurofilament medium, Nfl: Neurofilament low.

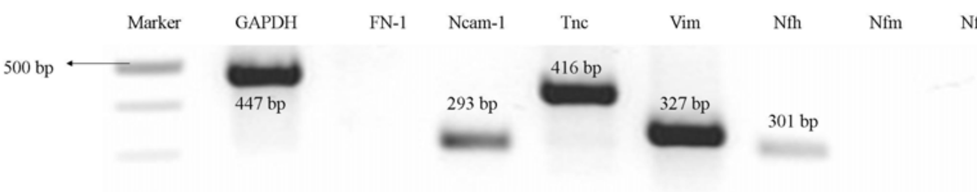

Fig. 4 Gene Expression of rF-98 by DNA electroforesis. Marker, GAPDH, FN: Fibronectin, Ncam 1, Tnc: Tenascin, Vim: Vimentin, Nf h: Neurofilament high, Nf m: Neurofilament medium, Nfl: Neurofilament low.

Brain mice E-10 were collected from each of 5 pregnant mice. The electrophoresis show the level of gene expression of cDNA brain embryo at gestation days (GD-10) mouse HT-22, rF98 and hLN-405. For the Fibronectin, Tenascin, Vimentin, NCAM gene expression from brain GD-10 (E-10) were not different with gene expression of mHT-22, and rF-98, except in FN-1 of hLN-405 is differ. Result of real time PCR gene expression of Neurofilament embryonic day 10 is differed from gene expression of mouse HT-22, rF-98 and hLN (Figure 1).

Stewart and Pearlman (1987) showed that the expression of fibronectin first appears in the neuroephitelial as small points of immunofluorescence among the earliest postmitotic that form preplate embryonic day 11 and 12. In this research the expression of the fibronectin was a little expressed. This suggests that cells in neuroephitelial that have been proliferated, will express vimentin, not fibronectin. Thus vimentin is very high expressed. Fibronectin plays a role in forming the migratory pathway for the growth cones of these axons. Sheppard *et al.,* (1995), showed that expression mRNA Fibronectin is high

deep in the neuroephitelial proliferation zone that contains dividing cells and the cell bodies of radial glia. Expression in the cortical proliferative zone is limited to the period of neurogenesis. Thus FN1 may be involved initially in supporting the cell division and fate determination that takes place in the neuroepithelium; later production by migrating neurons may play a role in the selection of radial glial pathways that lead to specific cortical regions, and in interactions between neurons as they form cortical layers within these regions.

In brain tissue, vimentin is detectable as early as embryonic day 11, the earliest stage and is located in radial fibers spanning neural tube, in ventricular cells (Schnitzer *et al.,* 1981). Vimentin has been detected in Brain embryonic day 10 and also at cells line mHT-22, *h LN-405, rF98.* The Vimentin is expressed in many cells of neuroectoderm in the fetal central nervous system (Sarnat, 1998). The neuroepithelial cells in the neuroectoderm, which constitute the primordium of the CNS, are potentially capable of generating neuronal and glial cell lineages concomitantly. The appearance and morphological development of vimentin-positive in neuroepithelial cells in human embryonic and fetal brain on 4–16 weeks (Stagaard and Mollgard, 2004). In embryos aged 4-6 weeks, vimentin-reactivity was seen in all neuroepithelial cells, including those which exhibited motitic figures. All regions exhibited vimentin-positive neuroepithelial cells, the distribution and morphology of which gradually changed, resulting in lamination of the neural wall into two and subsequently three layers. Vimentin is a marker for cell lineage during early central nerve system development. It is suggested that all neuroepithelial cells differentiate to a stage where they express vimentin and that vimentin may have a role in cellular movements and migration. Vimentin is 57 kDa intermediate filament cytoskeleton protein widely expressed in immature cells, including those of the human fetal brain, that changes with maturation. Vimentin is synthesized early in mammalian embryogenesis, including derivates of the neuroectoderm. The formation of vimentin is between days 7 and days 11 of mouse embryogenesis.

Cells hLN-405 (human, glioblastoma-astrocytoma) are cells culture, which used in this experiment. Astrocytomas are tumors that arise from brain cells called astrocytes. Glioblastoma originate from glial cells, most often astrocytes. Sometimes the terms “astrocytoma” and “glioma” are used interchangeably. Here, we use “astrocytoma” to refer to these tumors as a group. rF98 (rat glioma cell), is also group of glial tumor. Both of cell express high level of vimentin although are not neural cell.

The hippocampus is a major component of the brains of humans and other vertebrates. Hippocampus cell is neural cell, that express high level of vimentin compare to the another neural cell in the fetuses brain at GD-10.

Real time PCR also showed that the expression of hLN-405 cell (human glioblastoma-astrocytoma) was high compare to the other protein. Vimentin may involve in the signal response of the cell damage. When damage was occur in the brain, astrocyte active to expressed Vimentin. The high level expression may caused by as a pro-inflamsi signal, to improve recovery (Vaknin *et al*., 2002). Intermediate filaments (IFs) are highly diverse intracytoplasmic proteins within the cytoskeleton which exhibit cell type specificity of expression. A growing body of evidence suggests that IFs may be involved as collaborators in complex cellular processes controlling astrocytoma cell morphology, adhesion and proliferation. Vimentin was expressed by all astrocytoma cell lines. may serve as a marker for an astrocytoma cell type with enhanced motility and invasive potential (James et al., 1999).

4. CONCLUSION

In conclusion, the data gained from this research is the information of fibronectin expression development at gestation days 10. The fibronectin expression is lower than the vimentin expression, because in the earliest stage of cortical development of brain, FN is produced by cells in the ventricular zone. Thus after the proliferation stage in mice brain, fibronectin detectable was very low, because this protein has not being synthesized again. It is as early as embryonic day 11. The data based on real time PCR that showed vimentin expression is higher than the other protein expression at embryo gestation days 10. This vimentin played a role in neurogenesis because of its co expression with neurofilament in neuroblast.

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