EXPRESSION OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) AND AUTOTAXIN (ENPP2) IN TWO PHENOTYPICALLY DIFFERENT NEUROBLASTOMA CELL LINES

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Abstract

Neuroblastoma, one of the most common solid tumors of pediatric population, is a tumor of the peripheral autonomic nervous system. Neuroblastoma cells have spontaneous morphologic differentiation with three distinct cell phenotypes- 1) S- type or Schwannian / glial cells that are substrate-adherent and non-tumorigenic, 2) I-type or intermediate cells are highly invasive and malignant and 3) N- type or neuroblastic cells. I- type cells are multipotent embryonic precursors and capable of either neuronal or non-neuronal neural crest cell differentiation. Epidermal Growth Factor Receptors (EGFRs) are associated with survival, growth and cell proliferation in normal and malignant cells. Higher level of EGFR expression has been found to be associated with different types of cancers. Expression of EGFR mRNA in S and I-type neuroblastoma cells was studied by RT-PCR analysis. In addition, an mRNA that encodes human Autotaxin or Ectonucleotide pyrophosphatase phosphodiesterase (ENPP2), a mitogen responsible for cell motility and angiogenesis of tumor cells, was studied in I and S-type cells. While the EGFR encoding mRNA was found to be expressed in both S and I- type cells, with no significant difference in the expression level, ENPP2 mRNA was found to be expressed only in I but not in the S-type cell line.

Keywords: Neuroblastoma, EGFR, Autotaxin, ENPP2,

Introduction:

Neuroblastoma is the most common extra-cranial neoplasm in young children. It arises from neural crest cells and is most commonly present in the adrenal medulla or along the sympathetic chain (Ho et al., 2002). One of the most important features of this tumor is that its differentiation is diverse (Biedler et al., 1997) and it shows heterogeneity, both clinically and biologically (Ho et al., 2005). The majority of these tumors is generally metastatic at the time of diagnosis and thus have poor prognosis. Tumor histology is considered as one of the important criteria for diagnosis of neuroblastoma (Biedler et al., 1997). Histopathological examination has revealed that neurblastoma cells can be categorized into 3 distinct types: 1) Neuroblastic or (N) type cells with neuritic processes, neurofilaments , scant cytoplasm which grow as poorly attached aggregates, 2) Substrate-adherent or (S) type cells that are large, flattened cells with extensive cytoplasm and vimentin and are devoid of any cell processes, 3) Intermediate or (I) type cells expressing morphologic and biochemical traits intermediate between N and S cells (Biedler et al., 1997). I-type cells attach equally well to the substrate and to the cells, have more cytoplasm than N cells and have short neuritic processes. I-type cells possess cytoskeletal proteins and cell surface receptors common to both S and N-type cells. Tumorigenicity studies by Ross et al. (2003) focused on the colony forming ability of N, S and I cell lines in soft agar and formation of tumors in nude mice. Their study showed that S cells are non tumorigenic, forming very few or no colonies in soft agar and no tumors in nude mice. N cells formed tumors in athymic mice at a frequency of 33-100% and showed 4-32% colony forming ability. I-type cells have the highest malignancy among all three cell types forming tumors in 100% of mice and having 3 fold higher plating efficiencies than N type cells (Ross et al., 2003). N and I cells show anchorage –independent growth

whereas the S cell type is highly contact-inhibited, forms a contiguous monolayer and tend to have finite life span (Biedler et al., 1997). I- type cells show bidirectional differentiation to either N or S- type cells when induced by specific agents (Walton et al., 2004). Hence I-type cells are considered as malignant stem cells from which S and N-type cells can arise (Biedler et al., 1997).

Tyrosine kinase receptors have significant function in survival, growth and differentiation of normal and malignant cells. Previous studies suggested that receptor Tyrosine kinases have an important role in the behavior of both favorable and unfavorable neuroblastomas (Ho et al., 2005). Epidermal Growth Factor Receptor (EGFR) is a receptor tyrosine kinase that belongs to erb-B family of four closely related cell membrane receptors, namely, EGFR (HER 1 or erbB 1), erbB 2 (HER 2), erb3 (HER 3) and erbB4 (HER 4). HER 1 or EGFR is normally expressed in all epithelial and stromal cells, but mostly expressed in glial and smooth muscle cells (Ho et al., 2005). High expression of EGFR had been observed in different types of cancers like-gastric, colo-rectal, ovarian, breast and lung cancers (Ho et al., 2005). The EGFR has intracellular tyrosine kinase domain for signal transduction. Activation of EGFR occurs with the binding of the ligands (like epidermal growth factor, transforming growth factor etc) to the extra cellular domain of the receptor. This results in receptor dimerization that leads to intrinsic protein tyrosine kinase activity and tyrosine autophosphorylation (Sako et al., 2000). These events cause phosphorylation of several intracellular signaling proteins, leading to mitogenic signaling and other cellular activities (Alroy et al., 1997), (Riese et al., 1998). Also, EGFR signaling impacts tumor growth progression, cell proliferation,

angiogenesis, invasion/metastasis and inhibition of apoptosis (Baselga, 2000) (Wells, 2000).

Autotaxin (ATX), also known as Ectonucleotide pyrophosphatase/phosphodiesterase (ENPP2) is a glycoprotein and potent tumor cell motility–stimulating factor (Kishi et al., 2006). Autotaxin is synthesized by a variety of normal cells and tissues (Jansen et al., 2005). The expression of Autotaxin (ENPP2) is increased in various malignancies, including non-small cell lung cancer, breast cancer, renal cancer, thyroid cancer (Kishi et al., 2006) and some neuroblastomas (Kawagoe et al., 1997). Autotaxin has a cell proliferation- stimulating activity (Umezo-Goto et al., 2002), tumor cell motility-stimulating activity and angiogenic factor like activity (Nam et al., 2000), thus conferring tumorigenic and metastatic potential to cancer cells (Kishi et al., 2006). Autotaxin acts as an extracellular lysophospholipase D and hydrolyses lysophosphatidylcholine into lysophosphatidic acid (LPA) and choline (Umezo-Goto et al., 2002) and this LPA in turn is responsible for stimulating 1) cell proliferation by *ras* activation, 2) cell survival by PKB activation, 3) cell rounding by RhoA GTPase, and 4) cell migration by Rac GTPase (Jansen et al., 2005). By acting as an autocrine –motility factor and an angiogenic factor, autotaxin is responsible for tumor cell motility and aggressiveness in advanced stages of cancer (Nam et al., 2000). Due to its extracellular action and stimulation of metastatic cascade at multiple levels, Autotaxin (ENPP2) has been an attractive target for cancer treatment (Jansen et al., 2005). In fact, Autotaxin is considered to be a potential diagnostic marker for Glioblastoma mutliforme, a very aggressive and malignant cancer (Kishi et al., 2006).

The purpose of this project was to explore expression level of EGFR and Autotaxin (ENPP2) in two phenotypically distinct neuroblastoma cell lines through RT-PCR. The results of this study can provide the scope for future research on the use of the expression of these genes as a possible marker for neuroblastoma and as a potential target for antitumor therapy for the patients suffering from neuroblastoma.

Materials and Methods

Cell lines and their phenotypes

Neuroblastoma cell lines SH-EP1 and SK-N-ER were kindly provided by Dr. Robert Ross, Barbara Spengler and Leelesha Samarweera. The cells were cultured in MEM with 10% Fetal Calf Serum, 10µg/ml streptomycin and were incubated at 37°C with 5% carbon dioxide and humidity. The cell lines used and their phenotypes are provided in Table 1.

Cell lines studied	*Phenotypes*
SH-EP1	S
SK-N-ER	I

Table 1. *Phenotypes of the cell lines used in the project*

Total RNA Extraction

Total RNA from the two neuroblastoma cell lines was isolated using Ambion RNAqueous total RNA isolation kit following manufacturer's protocol with minor modifications. The trypsinized cells were treated with 700 µl of Lysis/Binding Solution and vortexed to obtain a homogenous lysate. To the lysate 700µl Ethanol was added. The lysate-ethanol mixture was applied to a Filter Cartridge and centrifuged at 15,000g for 1min. The flow through was discarded. 700µl of Wash Solution#1 was then added to the

filter cartridge and centrifuged at 15,000g for 1 min. In the next step 500µl of Wash Solution # 2 was added and centrifuged at 15,000 g for 1minute and this step was repeated once again. Then the filter cartridge was placed in a new collection tube and RNA was eluted by centrifugation with 30µl deionized water, preheated to 75°C. Extracted RNA was then stored at -80°C.

Primers

Specific primers were designed based on the reported human *EGFR* mRNA sequence (GenBank Accession Number NM_005228.3) and spanned exons 27-28. The forward and reverse primers used were *5' CTTGCAGCGATACAGCTCAG 3'* and *5'GGCTGTCGAATTGCTGTTG 3'*. Specific primers for human *ENPP2* mRNA sequence (GenBank Accession Number NM_006209.3) spanning exons 12-16 were designed. The forward and reverse primers were *5'GCTAAGAGACCTAAGAGGAAG 3'* and *5'TGGCATAGTGCAAACGTTTGG 3'*. As a loading control for this study, GAPDH was used. The primers for GAPDH were designed to produce a PCR amplified cDNA of 228 bp. Information regarding the primers is provided in the Table 2.

Gene	*Primers*	*Sequence*	*Exons involved*	*Expected PCR product size*
EGFR	Forward	5'CTTGCAGCGATACAGCTCAG 3'	27-28 (nucleotides: 3441 to 3706)	265 bp cDNA, 2895 bp genomic DNA
	Reverse	5' GGCTGTCGAATGTGCTGTTTG 3'		
ENPP2	Forward	5'GCTAAGAGACCTAAGAGGAAG 3'	12-16 (nucleotides: 1089 to 1551)	462 bp cDNA, 1.2 kb genomic DNA
	Reverse	5'TGGCATAGTGCAAACGTTTGG 3'		

Table 2. *Primers used in this project*

The primers were solubilized in distilled water and then diluted to a concentration of 10pmoles/µl which was the working stock.

RT-PCR

RT- PCR was performed on RNA extracted from neuroblastoma cell lines using the QIAGEN One Step RT-PCR Kit. 15µl of RT-PCR reaction samples were prepared that contained RT 5X buffer, dNTP mix, RT enzyme mix, RNAse free water, forward and reverse primers of EGFR, ENPP2, GAPDH and also RNA from S and I cell lines. Negative controls included substitution of RNA with RNAse free water. 20µl of mineral oil was added in each RT-PCR tube. The PCR tubes were placed in a thermal cycler and heated according to the protocol as follows: 50°C for 30mins for the reverse transcription, 95°C for 15 mins for the activation of Taq DNA polymerase, followed by PCR at 94°C for 30secs (denaturation), 58°C for 30 secs (annealing), 72°C for 30secs (extension) followed by a final extension of 72°C for 7 mins and a hold of 4°C. For detection of EGFR encoding RNA, the amplification was performed for 50 and 40 cycles. For detection of the ENPP2 encoding RNA, amplification was performed for 50 and 45 cycles. The same RT-PCR protocol was followed for the amplification of GAPDH, which was used as a loading control. After the completion of RT-PCR reaction, 2µl of loading dye was added to each PCR amplified sample. 7 µl of each sample was then run through 1.5% agarose gel containing ethidium bromide. Electrophoresis was run at 136 volts for 45 minutes and the PCR products were visualized under UV light.

RT-PCR product Purification

RT-PCR products were purified using Rapid PCR Purification Systems (Marligen Biosciences Inc.) following manufacturer's protocol with some modifications. To the amplified PCR products 400µl of Binding Solution was added and mixed thoroughly. The samples were then loaded into filter cartridges and centrifuged at 12,000g for 1min. 700µl of Wash Buffer containing ethanol was then added to each filter cartridge and centrifuged again at 12,000g for 1 min. After discarding the flow-through, a third centrifugation was done at 12,000g for 1 min. DNA in each cartridge was then eluted with 20µl of deionized water warmed at 70°C, followed by incubation at room temperature for 1 min. and then centrifugation at 12,000 g for 2 minutes to isolate the purified products.

Sequencing

RT-PCR purified product was then diluted to get a DNA concentration of 5-15 ng/µl in water. 8µl(80ng) of the diluted, purified DNA (amplified by EGFR primers) was mixed with 0.8µl of EGFR forward primer and another 8µl PCR product (amplified by EGFR primers) was mixed with 0.8µl of EGFR reverse primer.

For ENPP2, 8µl of PCR purified DNA was added to 0.8 µl of ENPP2 forward primer and another 8µl of PCR purified product was mixed with 0.8 µl of ENPP2 reverse primer. The samples were then added to separate wells of a 96 well plate and sent out for sequencing to Genewiz.

Results

The level of expression of the genes - EGFR and ENPP2 in two phenotypically different S and I-type neuroblastoma cell lines was studied by RT-PCR using specific primers for EGFR and ENPP2 genes. RT-PCR products were then fractionated in a 1.5% agarose gel in the presence of ethidium bromide and visualized under UV light. The products were then purified and sequenced.

For analysis of the EGFR gene product, RT-PCR was performed for 50 and 40 cycles. At both levels of amplification, EGFR encoding RNA was found to be expressed equally in the 2 cell lines. GAPDH, the loading control was found to be expressed at the same level in both the cell lines. Figure 1 shows the expression pattern of EGFR gene in S and I-type cells after 40 cycles of amplification.

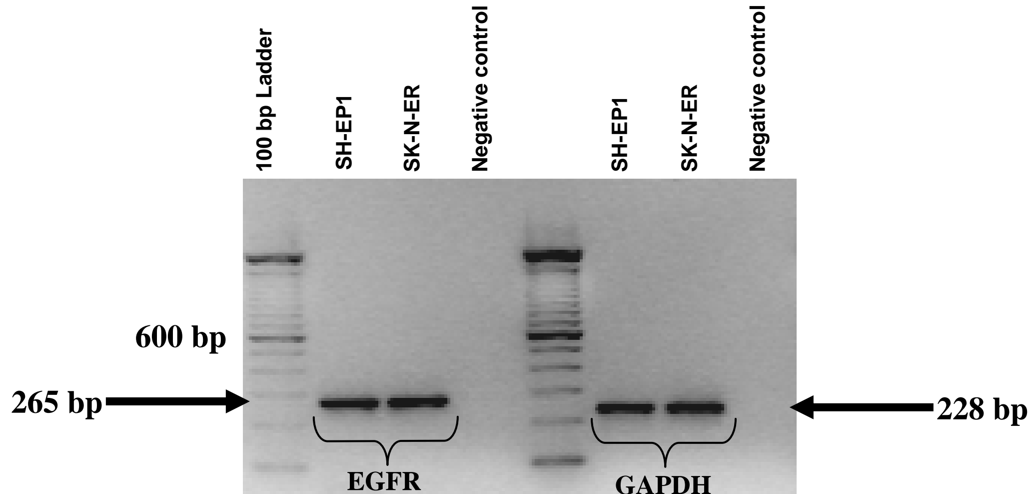

Figure 1. **Expression of Epidermal Growth Factor Receptor (EGFR) encoding RNA in 2 different neuroblastoma cell lines-SH-EP1 (S-type) and SK-N-ER (I-type).** EGFR primers were located in exons 27-28 and were designed to amplify a fragment of 265 bp. GAPDH was run as a loading control and produced an amplified segment of 228 bp.

For analysis of ENPP2 gene expression, RT-PCR was performed for 50 and 45 cycles and at both levels of amplification, ENPP2 was found to be expressed only in I- type and not in S- type cells. The loading control GAPDH revealed the use of equal amounts of RNA in these samples (Figure 2).

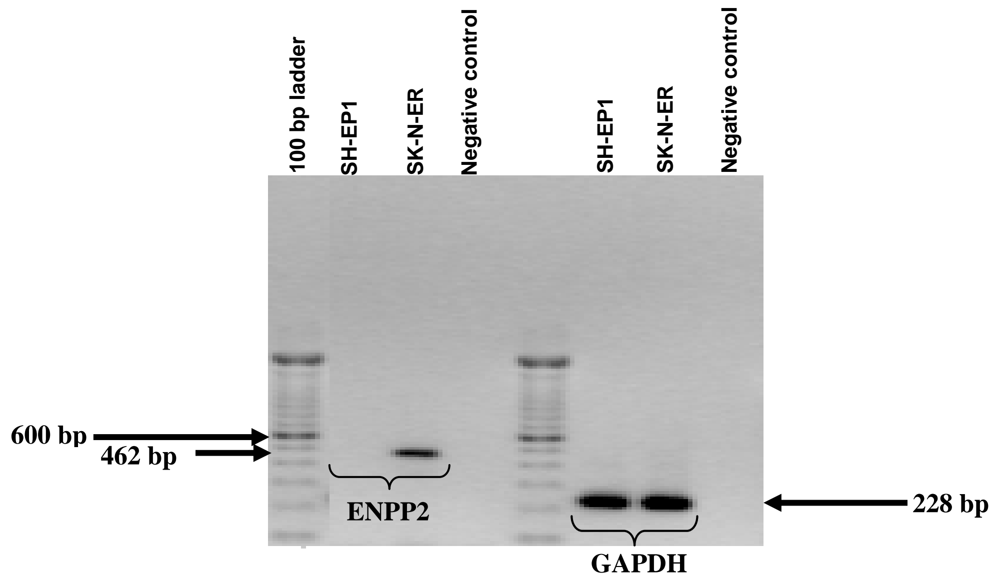

Figure 2: Expression of Autotaxin (ENPP2) in 2 different neuroblastoma cell lines- SH-EP1 (S-type) and SK-N-ER (I-type).ENPP2 primers located in exons 12 to 16 were designed to amplify a segment of 462 bp. GAPDH was run as a loading control and the primers were designed to amplify a segment of 228 bp.

In order to confirm the sequence identity of 265 bp PCR product (amplified by EGFR primers) and 462 bp PCR product (amplified by ENPP2 primers) the sequencing data of the PCR products obtained from Genewiz was aligned with the reported human EGFR mRNA and human ENPP2 mRNA sequences from NCBI. The blast comparison of about 160 bp sequence derived from EGFR (Figure 3) and 160 bp sequence from ENPP2

(Figure 4) showed 99% and 100% sequence homology, respectively, with the reported EGFR and ENPP2 mRNA sequences.

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RT-PCR
product    AAAGNGCCCGACCTGGCTCTGTGCAGAATCCTGTCTATCACAATCAGCCTCTGAACCCCG   60
           |||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
EGFR mRNA  AAAG-GCCCGACCTGGCTCTGTGCAGAATCCTGTCTATCACAATCAGCCTCTGAACCCCG   3598

RT-PCR
product    CGCCCAGCAGAGACCCACACTACCAGGACCCCCACAGCACTGCAGTGGGCAACCCCGAGT   120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EGFR mRNA  CGCCCAGCAGAGACCCACACTACCAGGACCCCCACAGCACTGCAGTGGGCAACCCCGAGT   3658

RT-PCR
product    ATCTCAACACTGTCCAGCCCACCTGTGTCAACAGCACATT   160
           ||||||||||||||||||||||||||||||||||||||||
EGFR mRNA  ATCTCAACACTGTCCAGCCCACCTGTGTCAACAGCACATT   3698
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Figure 3: Partial sequence alignment of 265 bp product with reported EGFR mRNA (NCBI Accession Number NM_005228.3). About 160 nucleotides of the purified RT-PCR product shows sequence homology with the reported EGFR mRNA.

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RT-PCR
product     TGGATGGACTGAAACAACTAAAACTGCATCGGTGTGTCAACGTCATCTTTGTCGGAGACC   60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ENPP2 mRNA  TGGATGGACTGAAACAACTAAAACTGCATCGGTGTGTCAACGTCATCTTTGTCGGAGACC   1320

RT-PCR
product     ATGGAATGGAAGATGTCACATGTGATAGAACTGAGTTCTTGAGTAATTACCTAACTAATG   120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ENPP2 mRNA  ATGGAATGGAAGATGTCACATGTGATAGAACTGAGTTCTTGAGTAATTACCTAACTAATG   1380

RT-PCR
product     TGGATGATATTACTTTAGTGCCTGGAACTCTAGGAAGAAT   160
            ||||||||||||||||||||||||||||||||||||||||
ENPP2 mRNA  TGGATGATATTACTTTAGTGCCTGGAACTCTAGGAAGAAT   1420
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Figure 4: Partial sequence alignment of 462 bp product with reported ENPP2 mRNA (NCBI Accession Number NM_ 006209.3). About 160 nucleotides of the purified RT-PCR product shows sequence homology with the reported ENPP2 mRNA.

Discussion

The study showed that EGFR mRNA is expressed in both S and I- type neuroblastoma cell lines with almost no significant difference in the expression level. Sequence analysis of the 265 bp RT-PCR product amplified by specific primers for human EGFR revealed almost 100% sequence homology with the reported human EGFR. EGF/EGFR pathway has been found to be an important pathway for the development of neuroblastoma. Receptor –ligand binding leads to the activation of intracellular signaling via MAPK and P13K pathways that could lead to enhanced growth, proliferation and survival of neuroblastoma cells (Ho et al, 2005). Previous study by Biedler et al. (1997) had shown the presence of EGFR protein in both S and I cell lines. Neuroblastoma consists of neuronal (N) cells, non-neuornal Schwannian or glial (S) cells and intermediate or (I) cells. I-type cells have many cell surface receptor proteins, cytoskeletal proteins and marker proteins common to both S cells and N cells (Biedler et al., 1997). This indicates that I- type is a stem cell precursor that gives rise to more differentiated N and S cell types. According to Ho et al. (2005), EGFR is highly expressed by glial cells, thus the expression of EGFR in S cells observed in this study provided evidence that S cells represent nonneuronal neural crest precursor cells that can give rise to Schwann, or glial cells. On the other hand, expression of EGFR by I-type cells show that they are intermediate cells expressing some cell surface receptor proteins similar to S cells. According to Biedler et al. (1997), the expression level of EGFR protein is highest in S-type, medium in I-type and very low in N-type. The present study indicates no significant difference in the expression level of the EGFR mRNA in the S and I cells analyzed. However, even if the mRNA level is not significantly different in these 2 cell lines, there

can be differences in the expression level of protein due to several reasons. Protein expression studies using Western blot analysis could be performed to assess EGFR protein levels in these cells. From the present study it appears that EGFR expression in neuroblastoma is not related to malignancy. However, analysis of larger sample size is required to reach any definitive conclusion.

In this study the mRNA for Autotaxin (ENPP2) was found to be expressed only in an I-type cell and not in a S- type cell. Sequencing data suggests a 100% homology between 462 bp RT-PCR product and the reported human ENPP2 mRNA. Autotaxin (ENPP2) is responsible for motility of the tumor cells by acting as a chemo- attractant. In a previous study, Kawagoe et al. (1997) indicated that N-type malignant neuroblastomas express Autotaxin that actually stimulates the motility of N- cells. Autotaxin, besides acting as an autocrine-motility factor also acts as an angiogenic factor and angiogenesis is required for growth and metastasis in different malignant tumors, including neuroblastoma (Chlenski et al., 2003). A large number of angiogenic stimulators appear to contribute the growth of clinically aggressive neuroblastoma tumors. On the other hand many angiogenic inhibitors help to maintain net inhibitory phenotype in Schwannian stroma-rich neuroblastoma, resulting in lower vascularity and allow the growth of neuroblastoma without angiogenesis (Chlenski et al., 2003). In neuroblastoma, I-type cells are detectable in tumors for all stages and the frequency of I cells is found to be higher in patients with progressive disease. I- type cells can thus be considered as prognostic of tumor progression (Ross et al., 2003). Hence the expression of ENPP2 mRNA in I- type neuroblastoma and not in S- type cell is in accordance to the findings of Nam et al.

(2000) who postulated that Autotaxin is responsible for causing aggressiveness in advanced stages of cancer.

Future research is needed to evaluate the expression level of Autotaxin mRNA and protein in larger number of neuroblastoma cell lines to determine the significance of the observation made by this study. This in turn can help to determine whether Autotaxin can be considered as a novel target for therapeutic intervention in neuroblastoma.

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