DETERMINING GENOMIC PROFILE AND APPLICATION IN TREATMENT OF NON-AMPLIFIED *MYCN* NEUROBLASTOMA PATIENT

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SUMMARY

Background: Neuroblastoma is the most common extracranial solid cancer of childhood and is characterized by a remarkable biological heterogeneity, cause multiple genetic changes. The genetic profiles are the powerful tools for the clinician in risk stratification and treatment tailoring in neuroblastoma patients. This will increase the chance of treatment's success and minimize the dose of chemotherapy for these patients. Subjects: 6 neuroblastoma patients under 18 months, non-amplified MYCN were diagnosed and treated in National Children's Hospital. Method: The CGH technique is performed on the Agilent's system with the 400k chip at Vinmec International Hospital. Results: 4 patients were found the numerical chromosomal abnormalities (both stage L2), the others were the segmental chromosomal abnormalities (1 stage L2 and 1 stage M). Based on this results, 4/5 patients could be stopped the chemotherapy, 1 patient had to continue the treatment. The stage M patient had the 50% of chance of success in high-dose chemotherapy and stem cell transplantation. Conclusion: The genomic profile by CGH is established successfully in Vietnam. The integration of this technique allows more precise prognostication and refined treatment assignment which contribute to improve survival with decreased toxicity.

* Keywords: Neuroblastoma; Genomic hybridization.

INTRODUCTION

Neuroblastoma (NBL), an embryonic tumour of the sympathetic nervous system, often affects children age 5 or younger [1]. It's the most common solid tumor in first year of life, with the prevalence approximately 1/7,000 live births. The median age at diagnosis is around 18 months [2].

Some specific genetic alterations in NBL had been discovered from 1980s, including the amplification of *MYCN* gene, gain 17q, loss 1p, loss 11p...

These genetic markers had provided more prognostic information, and contributed significantly in risk stratification and treatment tailoring in NBL patients. For example,

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the (near) triploid tumour has the good prognosis; or the amplified *MYCN* often occurs in high risk, worse prognotic patients [3]. Those aberations have divided into 2 separate groups: the numerical chromosomal abnormalities (NCA) and the segmental chromosomal abnormalities (SCA). The NCA tumour has found in infants, low stage, spontaneous regression and better prognosis case. Otherwise, the SCA profile, including the amplification of *MYCN* gene, alterations at 1p, 3p, 4p, 11q, 17q, exposure the worst prognostic for NBL patient [4].

The genetic alterations could be detected by classic karyotype or fluorescent in-situ hybridization (FISH) technique. While the karyotype shows time-consuming and low effective because of the requirement of metaphases from tumour cells, the limitations of FISH technique are expensive and low throughput. The apperance of array comparative genomic hybridization (aCGH), which has the posibility of whole chromosomes analysis, enabled the determination of genetic profile on NBL patients swiftly and high reliably. This profile have been used to classify NBL into risk groups based on the specific characteristics, corresponds with the diffenrent treatment plans and outcomes [4, 5].

The aCGH had been established in Untied States of America in 1992. Up to now, this technique had been optimized and became popular in genetic field. The first and most important component of aCGH technique is the DNA chip (or array), a region on the glass slide contains from thousands to millions distinct oligonucleotides (probes). Normally, the resolution using for NBL varies from 60,000 (60k) to 180,000 (180k) oligonucleotides per chip. The second component is the mix of 2 fluorescent DNA: target DNA dyed with Cy5 (blue) and control DNA dyed with Cy3 (dark pink), which have been put on the array to hybrid with the oligonucleotides. The ratio of fluorescent intensity displays the gain and loss at each probe position [6, 7].

At National Children's Hospital, there are 50 - 60 new diagnosed cases anually which investigate *MYCN* gene status by FISH technique for risk assessment. The low risk NBL (*MYCN* not-amplified) need the type of chromosomal alterations to choose the appropriate treatment protocol. Based on the collaboration between the National Children's Hospital, Vinmec International Hospital and Vinmec Research Institute of Stem Cell and Gene Technology, the study has been established for: *Either determining genomic profile on some NBL or tailoring the treatment in order to increase the chance of treatment's success and minimize the dose of chemotherapy for these patients.*

SUBJECTS AND METHODS

1. Subjects.

6 NBL patients in National Children's Hospital, under 18 months, without *MYCN* amplified have been selected from January to April 2017, including five L2 stage cases and one M stage case.

2. Methods.

* Samples:

Fresh tumour samples (not fix in formol) before chemotherapy is collected after the biopsy and store in -80°C until the test.

* aCGH technique:

The aCGH technique have been performed in Vinmec Research Institute of Stem Cell and Gene Technology on the Agilent system. DNA chip used was the SurePrint G3 Human CGH Microarray Kit, 2 x 400k (Agilent) with the resolution of 400,000 oligonucleotides covered 23 chromosomes.

The DNA was extracted by the kit of Qiagen Company and measured the concentration on the Nanodrop 2,000 (Thermo). Target DNA dyed with Cy5 and control DNA dyed with Cy3 were mixed and put on the slide, hybrid at $67^{o}C$ in 40 hours. The result has analyzed by CytoGenomics software (Agilent) with the helps from Curie Institute (Paris, France).

RESULTS AND DISCUSSION

1. Determination of genetic profiles.

The clear results enabled for analysis of genetic profiles accurately, in which 4 NCA cases and 2 SCA cases.

Table 1: List of NBL cases and the results.

Order	Labcode	Age at diagnosis	Stage	Genetic profile
1	NBL001	11 months	L2	NCA (-3, -4, +7, -10, -11, -13, -14, -16, +17, -19, -21)
2	NBL002	13 months	L2	NCA (-4, -5, +7, +8, -10, -14, -16, +17, +18, -19, -21)
3	NBL003	2 months	L2	NCA (-4, +7, -9, -10, -11, -14, -17, -21)
4	NBL004	15 months	L2	SCA (1p⁻, -4,9q⁻, 11q⁻, 17p⁻, 17q⁺, -19)
5	NBL005	12 months	L2	NCA (-4, -5, +7, +8, -10, -14, -16, +17, +18, -19, -21)
6	NBL006	12 months	M	SCA (1p⁻, 2q⁺, 3p⁻, 12q⁺, +13, 17q⁺, 19p⁻)

(-: Loss; +: Gain; p: Short arm; q: Long arm)

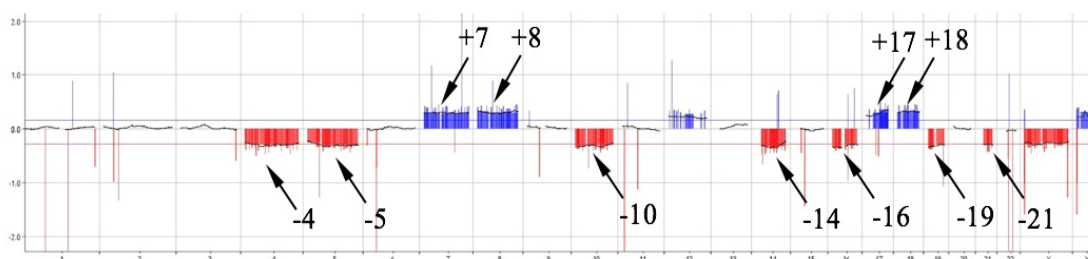Some genetic profiles on NBL were below.

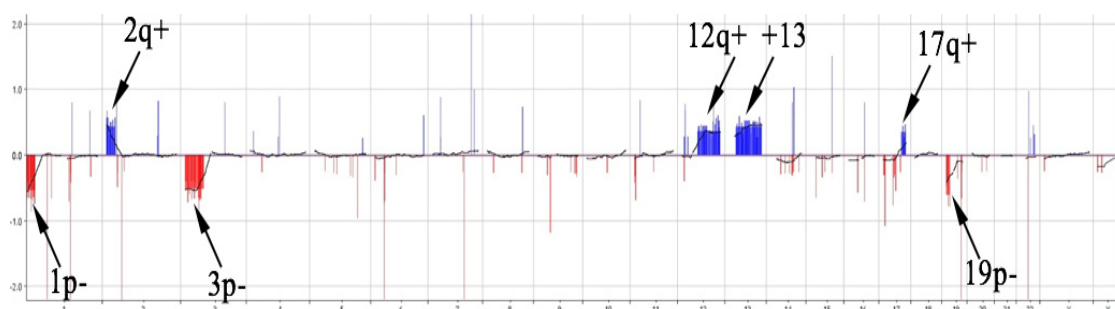*Figure 1:* The results of NBL005 patient (NCA type).

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Figure 2. The results of NBL006 patient (SCA type).

So, the genetic profiles of NBL had been well determined by aCGH, and beneficial in risk stratification and treatment plan.

2. Clinical significance in treatment tailoring.

The NBL patients in National Children's Hospital had been treated following the protocol of the International Society of Paediatric Oncology (SIOPEN). In five L2 stage NBL, 3 cases were unresectable and following-up after 2 courses of Carbo-VP16, 1 unresectable case after 3 courses of chemotherapy (2 courses of Carbo-VP16 and 1 course of CADO) and 1 new case. The decision of next chemotherapy courses depended on the genetic profile. If the genetic profile is NCA, the patient could be stopped chemotherapy and just follow-up. On the contrary, in case of SCA, the patient would be continued more 2 courses of chemotherapy.

Otherwise, the M stage patient had undergone the intensive chemotherapy based on the high risk treatment protocol, and now are having the palliative chemotherapy. The result of aCGH could change the future treatment plan, either draw up the chemotherapy (NCA type) or keep on the high dose chemotherapy, stem cell transplantation, surgery and radiotherapy with the successful rate of about 50% (SCA type).

The genetic profiles have assisted the clinical in tailoring the treatment in order to maximize the outcomes, specially in three L2 NBL: NBL002, NBL004 and NBL005. The NBL002 have abandoned the 4th course of chemotherapy (CADO) because of NCA type. The NBL004, a following-up case, by the SCA profile must be treated with 2 additional courses of chemotherapy and surgery for decreasing the risk of relapse. About the NBL005, this is a new NBL boy and the NCA profile help him avoid the chemotherapy while the size of tumor reduced by 40% in one month. Obviously, the determination of genetic profile by aCGH is the reliable tool, play an important role in risk stratification and treament tailoring.

CONCLUSION

The application of comparative hybridization technique in definition of the genomic profile has showed the clear

benefit on low-risk NBL patient, avoiding overtreatment or undertreatment for young patients. This is a grand step in developping the personalized medicine, resulting in high therapeutic effect as well as minimizing the complications of treatment for Vietnamese NBL patients.

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MUTATION ANALYSIS OF *EGFR* AND *FGFR* GENE IN GLIOBLASTOMA PATIENTS IN VIETNAM

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SUMMARY

Background: Glioblastoma is the most prevalence primary malignant brain tumor, which takes up 16% of all primary brain and central nervous system malignancy. Molecular variations or gene expression patterns have also been recognized in primary and secondary glioblastomas. Genetic typical alterations for primary glioblastoma are epidermal growth factor receptor and fibroblast growth factor receptors variations. Subjects and methods: We recruited 60 patients diagnosed with primary glioblastoma in which biopsy samples were collected to assess for FGFR and EGFR mutations. Results and conclusion: 6/60 patients (8.3%) were positive with FGFR mutation (p.R576W, p.A575V, p.N546K). 8/60 patients (13.3%) were identified with EGFR, a total of 7 mutations were identified p.P272S, p.G42D, p.T274M, p.K293X, p.L62I, p.G42D, p.A289T. This is the first study on FGFR and EGFR mutation in glioblastoma patients in Vietnam. The results would contribute to better understanding the pathological and molecular mechanism of glioblastoma in Vietnam.

* Keywords: Glioblastoma; EGFR; FGFR; Mutation analysis.

INTRODUCTION

Glioblastoma (GBM) is the most prevalence primary malignant brain tumor, which take up 16% of all primary brain and central nervous system malignancy [1]. The average age-adjusted incidence rate in the population is 3.2 per 100,000 [1]. GBMs were primary thought to be resulting exclusively from glial cells; however, recent studies suggest that they may result from several cell types with neural stem cell-like properties [2].

By the end of the genomic profiling and the Cancer Genome Atlas project (Parsons et al 2008), more than 600 genes were profiled from more than 200 human tumor samples, which revealed the complex genetic profile of GBM and we were able to characterize a set of three core signaling pathways that are commonly affected (i.e, the tumor protein p53 pathway, the receptor tyrosine kinase/Ras/phosphoinositide 3-kinase signaling pathway, and the retinoblastoma pathway) [3, 4]. Almost all primary and secondary GBMs presented abnormality in these pathways, allowing uncontrolled cell growth and persistence cell survival, while also letting the tumor cell to escape programmed cell death and

cell cycle checkpoint [5]. Molecular variations or gene expression patterns have also been recognized in primary and secondary GBM. Genetic alterations typical for primary GBM are epidermal growth factor receptor (EGFR) and fibroblast growth factor receptors (FGFRs) variations [4].

EGFR is a trans-membrane glycoprotein and belongs to the tyrosine kinase superfamily receptor [6]. Gliomas are tumors which emerge from glial cells, which express a variety of aggressiveness based on grade and stage. Many *EGFR* gene mutations have been characterized in gliomas, especially GBM. FGFR is a family of gene, sub-family of receptor tyrosine kinases (RTKs), it is comprised of four closely related genes (FGFR1-4) [7]. FGFR abnormalities have been associated with many cancers in human and play significant roles in tumor development and advancement [5, 7]. FGFRs activating mutations and overexpression have been linked with the development of various cancers, such as bladder, ovarian, breast, renal cell and more recently GBM [5, 8]. Up to now, there have been few studies to characterize mutation of FGFR and EGFR in Vietnamese patients with malignancy. This study aims: *To investigate the percentage and characterizes* EGFR and FGFR *gene alterations in GBM patients. The result will help better understand of the pathological and molecular characteristics of GMB in Vietnamese population.*

SUBJECTS AND METHODS

1. Subjects.

We recruited 60 patients diagnosed with primary GBM. Patients with secondary GBM or secondary tumor were excluded from the study. Informed consents were obtained from the patients prior to participation in the study. Biopsies taken from tumor-removing surgery were used to confirm diagnosis of GBM and for molecular investigation of *FGFR* and *EGFR* genes.

2. Methods.

* DNA extraction from biopsy sample:

DNA was extracted from biopsy sample using the phenol-cloroform-isoamyl method. DNA concentration and purity were verified using Nanodrop (ThermoFisher, US).

* FGFR and EGFR mutations analysis:

To identify point mutations in the *FGFR* and *EGFR* genes, another PCR amplification product (100 - 150 ng starting DNA) was obtained for each sample. After agarose gel discrimination, the PCR product was purified with Gel Purification Kit followed by sequencing using Big Dye Terminator V3.1 on ABI 3500 genetic analyzers (Applied Biosystems, CA, USA). Results were analyzed by CLC Main Workbench Software. Novel mutations were confirmed by conducting search on online databases (i.e. LOVD, 1000 Genomes, ExAC, and Pubmed) and all previous publications on *FGFR* or *EGFR* gene mutations. The primers used are provided by the author on reasonable request.

* In silico missense mutation analysis:

For novel missense variants, to predict whether the mutation has direct impact on EGFR or FGFR function, we utilized several in silico tool: Mutation Taster which estimates the pathogenic probability of DNA sequence change and predict the functional consequences of other non-coding

sequence or deletion/insertion mutations [6]; polyphen-2, a method using prediction models like HumVar and HumDiv for predicting damaging missense mutations. DUET to predict protein stability change upon mutation, results were taken from the mutation Cutoff Scanning Matrix (mCSM) method which calculate the mutated protein structure to be stabilizing or destabilizing.

RESULTS

1. FGFR mutation.

Table 1: FGFR mutation detected in the study cohort of 60 GBM patients.

Patient ID	Exon	Nucleotid change	Amino acid change	Publication
GB46	13	g.57835C>T	p.Ala575Val	Novel
GB48	12	g.56504C>T	p.Asp546Lys	Previously reported by Rand et al [9]
GB52	13	g.57837C>T	p.Arg576Try	Rand et al
GB53	13	g.57837C>T	p.Arg576Try	Rand et al
GB57	13	g.57837C>T	p.Arg576Try	Rand et al

Table 1 showed the result of FGFR mutation spectrum in 60 GBM patients in the study's cohort. After mutation analysis, 5/60 patients (8.3%) were positive with FGFR mutation. Of these, 2 mutations were located on exon 13 (1 mutation had been reported p.R576W, 1 with novel mutation p.A575V), 1 mutation located on exon 12 (p.N546K).

2. EGFR mutation.

Table 2: EGFR mutation detected in the study cohort of 60 GBM patients.

Patient ID	Exon	Nucleotid change	Amino acid change	Publication
GB6	7	c.814C>T	p.Pro272Ser	Rand et al
GB8	7	c.814C>T	p.Pro272Ser	Rand et al
GB10	7	c.814C>T	p.Per272Ser	Rand et al
GB23	2	c.124G>A	p.Gly42Asp	Rand et al
GB24	2	c.124G>A	p.Gly42Asp	Rand et al
	7	c.820C>T	p.Thr274Met	Rand et al
	7	c.877A>T	p.Lys293Stop	Rand et al
GB25	2	c.183C>A	p.Leu62Iso	Rand et al
GB26	2	c.124G>A	p.Gly42Asp	Rand et al
	7	c.866G>A	p.Ala289Thr	Rand et al
GB27	7	c.866G>A	p.Ala289Thr	Rand et al

Table 2 showed the result of EGFR mutation identification in 60 GBM patients in the study's cohort. After mutation analysis, 8/60 patients (13.3%) were identified with EGFR.

A total of 7 mutations were identified p.P272S, p.G42D, p.T274M, p.K293X, p.L62I, p.G42D, p.A289T. All mutations were previously reported in other studies.
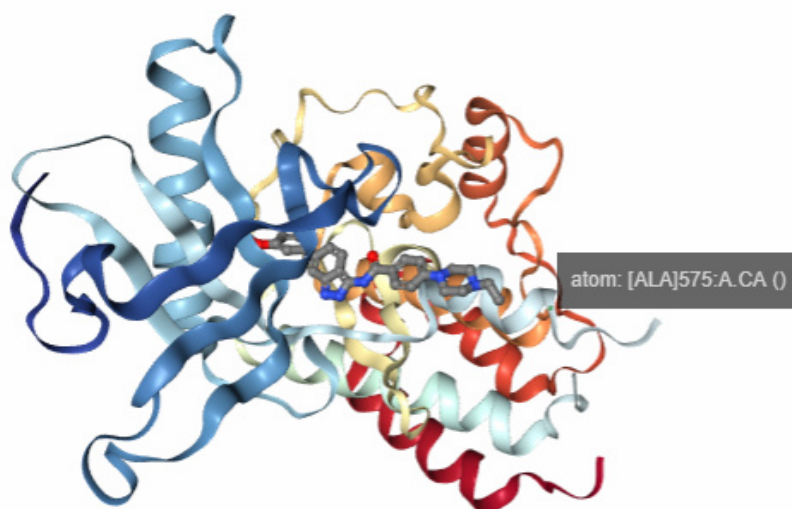

Figure 1: Molecular prediction model of novel mutation p.A575V.

Figure 1 showed the stimulated protein structure of FGFR with mutation p.Ala575Val. Prediction models (MutationTaster, Polyphen2, DUET) showed the mutation would cause altered FGFR activity thus contributes to the phenotype and neoplasticity of GBM.

DISCUSSION

The current study investigated the mutation spectrum of FGFR and EGFR in Vietnamese GBM patients. The patients had been enrolled and oncologists and pathologists carried out clinical evaluation to confirm the diagnosis of primary GBM. Therefore, the cohort is well defined and well suited for molecular study.

We identified FGFR mutation in 5/60 cases (8.3%), the mutation detection rate is comparable with other study in which FGFR mutations were identified in which it is higher than previously reported. Snuderl et al (2011) and Szerlip et al (2012), found that, FGFR mutations were found in 3 - 3.5% of cases [10]. The difference may be due to the difference in GBM staging between the cohort or the genetics composition of Vietnam compared to other population. The study identified 3 FGFR mutations, including 3 missenses p.R576W, p.A575V, p.N546K. 2 mutations (p.R576W and p.N546K) were previously reported. We identified a novel mutation p.A575V, we utilized prediction models (MutationTaster, Polyphen2, DUET) showed the mutation would cause altered FGFR activity thus contributes to the phenotype and neoplasticity of GBM. However, further *in vitro* and *in vivo* studies are needed to confirm the mechanism in which this mutation affects GBM pathogenicity.

We identified EGFR mutation in 8/60 cases (13.3%). Many EGFR modifications in gliomas have been reported in the literature, some of which were specific to GBM. EGFR amplification was seen in 0 - 4%, 0 - 33% and 34 - 64% of grade II, III and IV astrocytomas, respectively. 44% of patients with EGFR amplification had EGFR point mutations, mostly seen in the extracellular domain - e.g, A289 or R108 [11]. Other studies reported EGFR amplification in GBMs, anaplastic oligodendrogliomas (AOs) and anaplastic oligoastrocytomas (AOAs). EGFR overexpression was seen in 6 - 28%, 27 - 70% and 22 - 89% of grade II, III and IV astrocytomas, respectively, and represents an increase in gene transcription independent of DNA alterations. Half of the tumors with focal amplification and/or mutation of PDGFRA harbored concurrent EGFR alterations (14/33 patients = 42.4%), as did the majority of MET-altered tumors (3/4), reflecting a pattern of intratumoral heterogeneity that has been previously documented by in situ hybridization.

FGFR and EGFR are both potent oncogene; therefore, in many cases of malignancy there exist some form of mutation in these genes. The identification of FGFR and EGFR mutation has become routine in cancer management such as non-small cell lung cancer. In GBM, these genes have undergone extensive clinical trial for targeted therapy and for prognostic biomarkers [9]. FGFR mutation and fusion are undergoing trials for targeted therapy (TKI), and many mutation specific drugs are being tested. Similarly, the mutations have been linked with respond to erlotinib (first generation EGFR TKI) with prolonged survival and/or longer time to progression [12]. It is clear that FGFR and EGFR have been proven to be an independent factor in gliomagenesis and play a role in tumor formation. Although FGFR and EGFR status as a clinical marker remains controversy, more trails are needed to verify the clinical implication of each mutation. Finally, the need for larger study in Vietnam is required to examine the prognostic significance of *FGFR/EGFR* gene and protein status for survival, treatment and other clinical factors affecting the patient's outcome and quality of life.

CONCLUSION

This is the first study on FGFR and EGFR mutation in GBM patients in Vietnam. The results would contribute to better understanding of the pathological and molecular mechanism of GBM in Vietnam.

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