Scientific report: Identification of markers that can predict malignant behavior in pheochromocytomas and paragangliomas.

Exchange Grant 3824

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Host: Mercedes Robledo, CNIO

1) Purpose of the visit:

Pheochromocytomas (PCC) and paragangliomas (PGL) are rare tumors that occur at an incidence of 1 to 2 per 100,000 and 0.16 per 100,000 respectively. PCC arise from the chromaffin cells of the adrenal medulla and produce catecholamines, whereas PGL occur outside the adrenals and are subdivided into sympathetic (catecholamine-producing) and parasympathetic (biochemically silent) PGL. Both PCC and PGL occur in the context of several syndromes, including Multiple Endocrine Neoplasia type 2 (caused by germline *RET* mutations), Von Hippel-Lindau disease (caused by germline *VHL* mutations), Neurofibromatosis type 1 (caused by germline *NF1* mutations), and the PCC-PGL syndrome (caused by germline *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* mutations). Other genes that have been associated with PCC and/or PGL are *SDHA*, *TMEM127*, and *MAX*. Approximately 46% of the PCC and PGL bear somatic or germline mutations in one of the above-mentioned genes, but the underlying pathogenesis of the remaining tumors is still unclear. Approximately 10% of PCC and PGL develop metastases, which usually occur in the lymph nodes, liver, lungs, or bones. In fact, the frequency of malignancy is much higher in patients with an *SDHB* mutation (40%), or a sympathetic PGL (60%). Complete removal of the primary tumor is standard therapy for a benign PCC or PGL, but there does not exist a standard treatment for malignant PCC or its metastases. Patients with malignant PCC have a poor prognosis, with a 5-year survival rate of 32-60%. Many attempts have been made to identify molecular or immunohistochemical markers, or histological features that could predict malignant behavior of a PCC or PGL. However, until now this has not been successful yet.

The Fp7 ENS@T-CANCER project is a European network of 16 medical centers from 7 countries that studies adrenal tumors to improve diagnosis and treatment possibilities. The studies of work packages 2, 3, and 4 concern the investigation of the pathogenesis of ACC and PCC on a molecular and immunohistochemical basis, whereas the studies of work packages 5, 6, and 7 focus on improving diagnoses and functional imaging of these tumors. Our research group of the Erasmus MC, University Medical Center Rotterdam (the Netherlands), is mainly responsible for work package 3, which includes making tissue micro arrays (TMAs) of PCC and ACC, validating the previous published SDHB immunohistochemistry on a large series of tumors, and validating biomarkers that will be identified in work package 4. The identification of markers in work package 4 that can predict the biological behavior

and malignant potential will be performed in the Spanish National Cancer Center (CNIO) in Madrid (Spain), by the research group of Mercedes Robledo. This means there is a close collaboration between the investigators of work packages 3 and 4, as the markers that are found differentially expressed in the omic-studies, found by the research group in the CNIO will be validated at the Erasmus MC in Rotterdam. Therefore, this was the perfect opportunity for a (none-simultaneous) exchange visit between the two medical centers.

One objective of my work in Madrid was to find biomarkers for malignancy through analyzing and integrating the results from SNP arrays and messenger RNA profiling. A large part of these experiments had already been done, but the analyses had to be performed yet. In addition, I was involved in validation of the results from these studies. Another study in which I was participating, involved determining what chromosomal regions are of interest in their ability to predict malignant behavior. For validation, and to make a method that can easily be used in molecular diagnostics, we had to design a multiplex PCR.

In conclusion, the aim of my visit to the CNIO was to identify molecular markers that can predict malignant behavior in PCC and PGL, and to validate these markers with other techniques on independent series of tumors.

2) Description of the work carried out during visit

The work that was carried included analyzing mRNA expression data from benign and malignant pheochromocytomas, to identify markers that were differentially expressed between the two groups. To validate genes potentially related to malignant behavior, RNA was isolated from an independent series of cases collected by the other Centers involved in the Fp7 ENS@T-CANCER project. The RNA was used for the validation step by quantitative RT-PCR.

Second, SNP arrays had previously been performed on a large series of pheochromocytomas with different genetic backgrounds. The SNP data still had to be analyzed and validated. The most interesting result was the identification of nine chromosomal regions of gain that were related to malignant disease. To validate these regions, an assay was designed, based on labeled multiplex PCR. An independent series of DNA samples with clinical information was available for this validation experiment.

Third, there appeared an interesting mouse model, which had been created to study melanomas. However, the model had a leaky promoter, which caused the mice to develop the cardiofaciocutaneous syndrome, without presenting with melanomas. In contrast, the mice developed malignant pheochromocytomas, which metastasized to several organs, such as lungs, liver, pancreas, and testis. Since these metastases are relatively large it would enable us to perform molecular analyses on these tumors. This unique model would also enable to study different (chemo-) therapeutics; therefore we have written a project proposal which was submitted to the Association for International Cancer Research in the October round of 2012.

3) Main results obtained

Expression arrays

mRNA expression data from 40 benign and 12 malignant cases was analyzed using the freely available "R". Data was analyzed according Affymetrics recommendations. The "Affy" package was downloaded from www.bioconductor.com and the cell files were uploaded and expression data was normalized. Background was normalized through the RMA method, expression data was normalized through the quantiles method, and intensities were normalized through the perfect match method. The normalized results were included in one text file, which was used for further analyses. Merging probes from the same gene was performed using the freely-available babelomics (http://babelomics.bioinfo.cipf.es/). Statistically significant differences between malignant and benign tumors were analyzed by the freely-available pomelo 2 (http://pomelo2.bioinfo.cnio.es/). Fifteen genes that showed a significant fold change and had previously been reported to be involved in metastatic behavior were validated by qRT-PCR. Of these genes, MOXD1, TOP2A, and TIMP4 showed significant differences by qRT-PCR, and will be confirmed by immunohistochemistry at the Erasmus MC (Rotterdam, the Netherlands).

SNP arrays

Both Comparative Genomic Hybridization (CGH) as well as SNP arrays can be used to investigate chromosomal copy number alterations in tumors. One of the benefits of SNP arrays is the higher resolution compared to CGH arrays. In addition, this technique enables you to identify uniparental disomy, which recently has been shown to occur in *MAX*-mutated tumors. To investigate what genomic alterations occur in PCC of various genetic backgrounds, and to study the differences in molecular alterations between benign and malignant PCC, we performed SNP arrays on 95 PCC of several Medical Centers (CNIO, Spain; Erasmus MC, the Netherlands; University of Florence, Italy), of which the genetic background or the metastatic behavior are listed in Table 1.

Table 1. Clinical data of the PCC

	SDHB	SDHD	RET	NF1	VHL	TMEM127	MAX	Sporadic	Total
Benign	6	4	17	7	16	1	2	34	87
Malignant	2	-	-	-	-	-	1	5	8

SNP arrays consist of paired probes that reflect either the SNP (A allele) or the wild-type nucleotide (B allele). The output of the SNP data includes LogR ratios (total A and B expression levels), and B allele frequencies (BAF; the ratio of the B allele compared to the A allele). To analyze, normalize, and visualize our data we used the 'ASCAT' (allele-specific copy number analysis for tumors) algorithm, an "R" based

algorithm that also takes into account the tumor aneuploidy and the non-aberrant cell infiltration. The ASCAT application also includes an ASPCF algorithm, which segments and flattens the LogR and BAF ratios, enabling a smoothed view of the chromosomal alterations per tumor (Figure 1). These modified ASPCF-LogR ratios were used to identify overlapping regions of loss or gain between tumor samples within subgroups, which included tumors of identical genetic background or metastatic behavior.

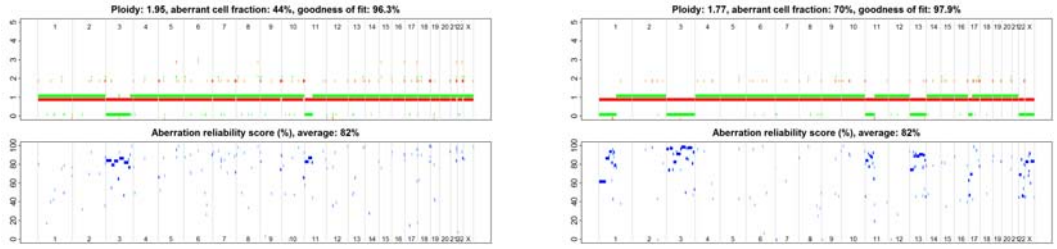

Figure 1 shows examples of ASCAT profiles of a VHL-mutated (left) and a RET-mutated (right) PCC.

The most common altered chromosomal regions of overlap are shown in Table 2A (showing common regions of overlap within the malignant PCC), and Table 2B (showing the chromosomal regions not altered in PCC). A selection of these chromosomal regions will be used for the validation of the SNP data. The overlapping regions per genetic background are not shown.

Table 2A

Loss of Chromosomal region	Basepair position (Mb) start	end
1p34.2-p31.3	42.4	62.1
1p31.3	65.8	68.3
1p31.1	76.6	68.3
1p31.1-p22.3	84	85.3
1p21.2	86.9	102.3
1p13.3-p11.2	107.6	121.3

Gain of Chromosomal region	Basepair position (Mb) start	end
4p16.3	0.5	1.1
7p22.3	0.4	1.6
8q24.3	144.3	145
9q34.3	138.9	140.8
10q26.3	134.6	135.5
14q32.33	104.6	107.3
16p13.3	0.6	2.3
17q25.3	78.9	81.1
20q13.33	60.6	62.9

Table 2B

Chromosomal region		Basepair position (Mb) start	end	No. of SNPS
Chromosome 5	5p15.2	10.3	10.6	80
	5p15.2	10.7	10.8	47
	5p15.2	14.1	14.2	30
	5p15.1	16.6	16.7	34
	5p13.1	29.4	29.5	27
	5q35.1	172.1	172.2	31
	5q35.1	172.34	172.43	35
Chromosome 12	12q14.1	60.9	61.1	32
	12q21.2	78.9	79	21
Chromosome 16	16p13.3	0.8	10.5	59
	16q23.3	82.2	82.3	46
	16q24.1	86.67	86.71	31
	16q24.1	87	87.1	49

Table 2A shows chromosomal regions that are altered, either lost (upper part) or gained (lower part), in more than 70% of the malignant tumors. Table 2B shows the selected chromosomal regions that showed no alterations in all of the PCC with known gene mutations, and the malignant cases. First, to decrease the amount of data load, a pre-selection was performed looking at the ASCAT profiles for chromosomes that rarely exhibit chromosomal alterations (<10% of all PCC). Second, after identification of these chromosomes, the ASPCF-LogR data was used again to find regions that never show alterations within groups of PCC with known gene mutations and the malignant PCC.

Figure 2.

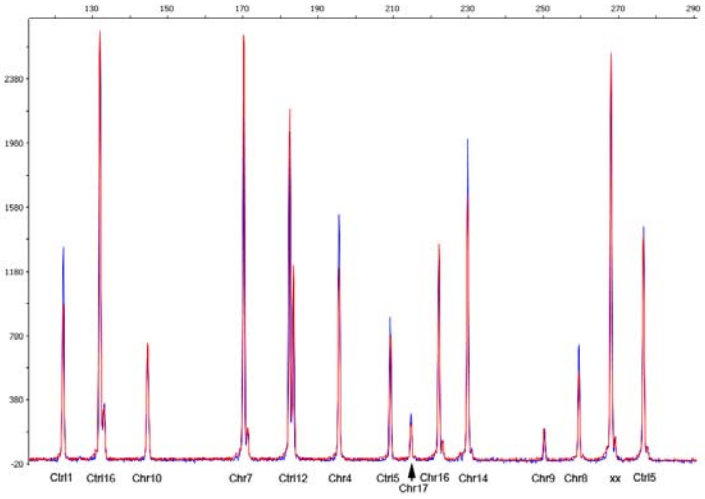

Figure 2 shows a electropherogram of a multiplex PCR result of normal medulla (blue) and tumor DNA (red). Control peaks, used to normalize the signals, were located on chromosomes 5, 12 and 16 (indicated by Crtl on the x-axe). The additional peaks include the peaks that showed gain in most malignant samples, with the exception of peak Ctrl1, which is a control peak for loss of chromosome 1.

A multiplex PCR was designed to enable validation of gains and losses in one experiment. For the multiplex PCR, primer combinations were created in the areas that were gained or lost at high frequency in the malignant tumors, and in areas that showed no alterations in any of the tumors according the normalized logR values. This technique has been - and still is - performed for examining germline DNA for large (exon) deletions. Freely-available Peak Scanner Software v1.0 (Applied Biosystems) was used to analyze and compare tumor and normal DNA samples. Although multiplex PCRs work perfectly on germline DNA, tumor DNAs showed too many genomic alterations, due to intra-tumoral heterogeneity, to provide us with valid results (Figure 2). Therefore, the SNP arrays will be confirmed by "Fluorescent In Situ Hybridization". This approach will be also performed in collaboration between the CNIO and the Erasmus MC.

Mouse model

During my stay, we learned about a *Braf* knock-in mouse model, which was created at the CNIO by the group of Mariano Barbacid, presenting with pheochromocytomas. It was even a more interesting model, because the mice had pheochromocytomas that metastasized to several organs, including the lungs, liver, pancreas, and testis. Although the frequency of mice displaying metastases was relatively not very high (16% of the total group), the metastases appeared to be much larger compared to the metastases found in the only two reported mouse models that have pheochromocytoma metastases. Since the size of the metastases is relatively large (up to 1 mm at the age of 30 weeks), it enables us to isolate sufficient amounts of DNA and RNA, so we could perform molecular experiments such as exome sequencing and expression arrays on them. Since this is the only mouse model that

presents with spontaneously developing pheochromocytoma metastases that metastasize so extensively, this would make it a very interesting model for testing different chemotherapeutics. In addition, it would also help us understanding the pathogenesis of malignant pheochromocytomas, and finding molecular markers for metastatic behavior.

Under supervision of Mercedes Robledo, I have written a project proposal to investigate the *Braf* mouse model in more detail. We submitted this grant proposal to the Association for International Cancer Research, for which the deadline was the 26th of October.

4) future collaboration

Future collaboration will include different studies that are included in the European Fp7 project. The mRNA expression study of the Fp7 project resulted in three genes that were significantly different between malignant and benign pheochromocytomas, validated in an independent series by qRT-PCR. These results will be validated by immunohistochemistry at the department of pathology of the Erasmus MC (Rotterdam, the Netherlands). Furthermore, the SNP array results need to be validated by Fluorescent In Situ Hybridization, which will be performed at the CNIO and the Erasmus MC.

A new subject of collaboration concerns the mouse model with metastatic pheochromocytomas. If our research proposal is granted, our research groups will be collaborating for the next three years. This will be an intensive collaboration, as breeding of the mice and a part of the molecular experiments will be performed at the CNIO, whereas the other part of the experiments will be performed at the Erasmus MC. The proposed project will involve many different collaborations with many scientists from different departments at the CNIO.

5) projected publications

This work will result in at least 2 publications of which I will be included as an author. The first paper will be about the mRNA expression, which will be written after the validation experiments (immunohistochemistry). The second publication will concern the genomic alterations between benign and malignant pheochromocytomas. The manuscript, of which I will be first author, will be written after the validation experiments (FISH analyses) have been performed. In addition, in an unrelated study, I assisted in evaluating immunohistochemical stainings for tyrosin kinase receptors of pheochromocytomas and paragangliomas, of which I will also be included as a co-author. Other projected publications will be about the studies on the *Braf* mouse model, if the project will be granted.

5) comments

Here, I would like to thank the European Science Foundation for creating this great opportunity of a six-month stay at the CNIO in Madrid. The ESF made it possible for me to stay for half a year in a prestigious and excellent scientific environment, in which I have learned so much. The scientists, PhD-students, and technicians at the CNIO are very collaborative, and I will work with many of them in future when our project will be granted. In addition, I would like to thank Mercedes Robledo for her hospitality and guidance, and I hope that our collaboration will continue for many years. Also, I would like to thank Ronald de Krijger, for giving me the time and opportunity to grow scientifically, by letting me stay at the CNIO for six months.