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Association of melanoma with intraepithelial neoplasia of the pancreas in three patients

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ABSTRACT

Melanoma and pancreatic cancer are two low frequency types of cancer. In this study, three patients who developed both melanoma and intraepithelial neoplasia of the pancreas were tested for *CDKN2A* mutations and deletions, and investigated for rare germline copy number variations (CNVs). The three patients were negative for *CDKN2A* point mutations and intragenic deletions. One of these patients carried two large (>300 kb) germline CNVs, both genomic duplications affecting coding sequences that are not copy number variable in the population. A second patient exhibited loss of the entire Y chromosome, an event probably coincidental related to his advanced age (79 years-old). Our data pinpoint that rare germline CNVs harboring genes can contribute to the cancer predisposition of melanoma and intraepithelial neoplasia of the pancreas.

Introduction

Pancreatic cancer has one of the highest death rates among all cancer types. The relative 5-year survival rate of pancreatic cancer in the USA was estimated in 6% (http://seer.cancer.gov/), primarily due to the late detection of the disease and the lack of effective treatment (Ma and Jemal, 2013).

It is already known that familial history of pancreatic cancer is an important risk factor associated with this tumor development (Permuth-Wey and Egan, 2009). Nevertheless, only 20% of all familial pancreatic cancer has a known genetic etiology. Mutations in some specific genes were already reported increasing the susceptibility to pancreatic cancer, such as *BRCA1*, *BRCA2*, *ATM*, *STK11*, *PRSS1*, and *PALB2* (Jones et al., 2009; Solomon et al., 2012). In addition, some previous studies have established that familial history of melanoma is a risk factor to the development of pancreatic cancer (Rustgi, 2014; Zavoral et al., 2011). Melanoma exhibits a low but increasing incidence (Jemal et al., 2006), and although accounting for only 4% of all skin cancer cases, melanoma is responsible for approximately 75% of all deaths caused by cutaneous neoplasias (Schinke et al., 2010). Among all melanoma cases, approximately 10% occur in a familial context, and up to 40% of the familial melanoma are caused by mutations affecting the *CDKN2A* gene, mainly the p16 variant (Pho et al., 2006). *CDKN2A* mutation is associated with the occurrence of pancreatic cancer in familial melanoma with a cumulative risk of up to 20% by age 75 (Rustgi, 2014). Few genes were associated with the concomitant development of melanoma and pancreatic cancer in the same patient, mainly the aforementioned *CDKN2A*, and also *BRCA1* (Bartsch et al., 2010; Solomon et al., 2012).

There are three precursor lesions giving rise to pancreatic cancer that includes pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), each of them arising in association with distinct genetic alterations (Macgregor-Das and Iacobuzio-Donahue, 2013).

Pancreatic intraepithelial neoplasia (PanIN) can be divided in three grades based on their degree of morphologic atypia and mucin output. The entire spectrum – PanIN-1, PanIN-2, PanIN-3 – represents a gradual neoplastic progression and cumulative alterations on gene expression, including somatic mutations on *KRAS2*, *TP53*, *CDKN2A*, and *MADH4*/*SMAD4*/*DPC4* (Biankin et al., 2003; Macgregor-Das and Iacobuzio-Donahue, 2013; Maire et al., 2013). PanINs are the most frequent pancreatic cancer precursor, even though it may arise from any of the three precursor lesions described above (Macgregor-Das and Iacobuzio-Donahue, 2013). In preinvasive IPMN, mutations in *KRAS*, *TP53*, *BRAF*, and in the serine/threonine kinase *STK11*/*LKB1* have been reported (Sahin et al., 2003; Schönleben et al., 2007). Mutations in *KRAS* and *TP53* have been also noted in higher-grade lesions of MCNs.

Germline copy number variations (CNVs) are gains and losses of large genomic segments composing with other variants the genetic background of human populations (Redon et al., 2006; Sebat et al., 2004). CNVs encompass approximately 15% of the human genome,

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and mainly affect a subset of genes involved in immunity, interaction to environment and infection (Li and Olivier, 2013). This type of genomic variants can modulate the phenotype and therefore contributes to the genetic individuality (Li and Olivier, 2013). Although most of the CNVs are not pathogenic, several were causally related to congenital diseases, and some were already associated with susceptibility to cancer, including melanoma and pancreatic cancer (Al-Sukhni et al., 2012; Gonçalves et al., 2012; Krepischi et al., 2012a, 2012b; Krepischi-Santos et al., 2006; Lucito et al., 2007; Shaw-Smith et al., 2006; Yang et al., 2012). Therefore, rare germline CNVs can harbor genes composing the genetic susceptibility to cancer. Genomic profiles of germline CNVs of three melanoma patients who also developed intraepithelial neoplasia of the pancreas were assessed in order to find rare variants possibly related to the phenotype.

Materials and methods

Study approval and patient samples

The patients were ascertained at the Skin Cancer Department of the A. C. Camargo Cancer Center, São Paulo, Brazil (ACCCC). This is a retrospective study approved by the local Ethics and Research Committee of the Institution (#1728/12), and signed informed consents were obtained from the patients. This patient group comprised 3 unrelated probands who developed both cutaneous melanoma and intraepithelial neoplasia of the pancreas. DNA samples were extracted from peripheral leukocytes following standard procedures.

CDKN2A and CDK4 sequencing

DNA sequencing was conducted at the Laboratory of Genomics and Molecular Biology (ACCCC). Direct sequencing of the *CDKN2A* ($p16^{INK4a}$ and $p14^{ARF}$) and the *CDK4* genes was performed on DNA samples extracted from peripheral blood (primer sequences available upon request). Variants of unknown clinical significance were investigated in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and 1000 Genomes (http://www.1000 genomes.org/).

DNA copy number variation (CNV) assessment

We performed the evaluation of CNVs using the CytoSNP 850K BeadChip (Illumina) which contains 850,000 SNP probes covering the whole-genome with enriched coverage for 3262 well-known genes of constitutional and cancer diseases. Labeling, hybridization, and washing procedures followed the manufacturer's instructions. Microarray slides were scanned using the iScan System (Illumina), and generated gtc files were loaded on the BlueFuse Multi Software v3.2 (BlueGnome) to evaluate experimental quality and CNV calling. Alterations had to encompass at least five consecutive probes with aberrant $\log_2$ values with a minimum size of 100 kb. The detected variants were compared to the CNVs' panel of normal populations reported in the Database of Genomic Variants (DGV; http://projects.tcag.ca/variation/; freeze January 2014), and common CNVs were disregarded. Variants presenting with frequencies <0.1% among the control individuals, and harboring genes (thereafter referred as rare CNVs) were selected for further validation. Gene annotation was performed using the University of California Santa Cruz Genome Browser (UCSC; http://genome.ucsc.edu/cgi-bin/hgGateway) and the Catalog of Somatic Mutations (COSMIC; http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Copy number validation by real-time quantitative PCR (qPCR)

In order to validate two rare CNVs, qPCR was performed using the SYBR Green system (Applied Biosystems) on a 7500 System apparatus (Applied Biosystems) with a reference DNA (Promega) as control for copy number calibration. We designed probes covering coding sequences of one of the genes contained in each CNV (Supplementary Table S1). Values were normalized based on data from the *GAPDH* (12p13) e *HPRT* (Xq26.2) genes as described by Torrezan et al. (2012). Triplicates were analyzed using the comparative $2^{-}\Delta\Delta C^{t}$ cycle threshold method (Livak and Schmittgen, 2001). Values in the range of 0.8–1.2 indicated two copies, <0.6 indicated copy number loss, and >1.4 was considered a gain.

Results

Clinical description of the three patients can be found in Table 1. These patients were screened for point mutations affecting the coding sequence, promoter or splice junctions of the *CDKN2A* gene, and no pathogenic mutations were detected. Additionally, we investigated the presence of *CDKN2A* deletions using the 850K SNP-array data, and copy number alterations affecting the *CDKN2A* genomic region were not detected.

Two rare germline CNVs validated by qPCR were detected in one of the patients: a 616 kb duplication at 2p12 encompassing the *CTNNA2* and *MIR4264* genes, and a 368 kb duplication at 5q35.3 containing the *GRM6*, *ADAMTS2*, *ZNF879* and *ZNF354C* genes. Genomic mapping of the variants are detailed in Table 2. These duplications occurred in genomic segments with few or none reports in the Database of Genomic Variants (Fig. 1).

Additionally, one patient who was 79 years-old at diagnosis was found to exhibit loss of the entire chromosome Y in leukocytes.

Discussion

Germline copy number alterations are already known to contribute to cancer susceptibility, including melanoma and pancreatic cancer (Al-Sukhni et al., 2012; Krepischi et al., 2012b; Lucito et al., 2007; Silva et al., 2012; Yang et al., 2012). A recent study identified a 4q13 germline duplication in one melanoma family (Yang et al., 2012). Concerning pancreatic cancer, three CNV studies provided candidate genes to

Table 1
Description of clinicopathologic features of the 3 patients regarding to melanoma and pancreatic cancer status.

Patient	Rare germline CNV	Actual age/gender	Melanoma clinicopathologic features (diagnostic age, location, histopathology)	Melanoma status disease	FMS	Pancreatic cancer clinicopathologic features (diagnostic age, symptoms, histopathology)	Pancreatic cancer status disease	FPCS
1	0	69 y/M	57 y, trunk, superficial spreading melanoma, B = 0.5 mm	AWD	No	68 y, asymptomatic, well-differentiated neuroendocrine tumor and IPMN	AWD	No
2	Duplications at 2p12 and 5q35.3	65 y/M	58 y, trunk, superficial spreading melanoma, B = 0.45 mm, CL II	AWD	No	64 y, abdominal pain, microcystic serous cystadenoma and PanIN-1	AWD	No
3	Chr. Y loss	85 y/M	78 y, trunk, lentigo maligno melanoma, B = 3.2 mm, CL IV, BRAF negative	Local and linfonodal metastatic	No	84 y, asymptomatic, invasive mucinous adenocarcinoma and IPMN	AWD	No

FMS — Familial Melanoma Syndrome; FPCS — Familial Pancreatic Cancer Syndrome; B — Breslow thickness; CL — Clarck level, PanIN-1 — low grade pancreatic intraepithelial neoplasia; IPMN — intraductal papillary mucinous carcinoma; AWD — alive without disease.

Table 2
Description of two rare germline copy number variations (CNVs) identified in one patient, with respective genomic positions (GRCh37), ISCN 2013 description, size, type of copy number event, and affected genes.

Cytoband	Start position	ISCN 2013	Size (kb)	CNV type	Genes affected[a]
2p12	79,539,758	arr 2p12(79,539,758-80,156,246)x3	616	Duplication	*CTNNA2*[a], *mir4264*
5q35.3	178,413,947	arr 5q35.3(178,413,947-178,782,813)x3	368	Duplication	*GRM6*[a], *ADAMTS2*, *ZNF879*, *ZNF354C*

[a] Genes selected for validation using qPCR.

pancreatic cancer susceptibility (Al-Sukhni et al., 2012; Huang et al., 2012; Lucito et al., 2007).

Although loss of Y chromosome has been associated with some tumors such as lymphocytic leukemia (Chapiro et al., 2014), it was already described in low percentage of men after the seventies (Guttenbach et al., 1995). The two detected CNVs were duplications, one of them affecting a 2p12 segment sizing 616 kb that harbors the *CTNNA2* and *MIR4264* genes. *CTNNA2* acts at cadherin adhesion that regulates cell–cell adhesion and differentiation (*Gene Cards* — www.genecards.org), and it was recently described as a tumor suppressor gene in laryngeal cancer (Fanjul-Fernández et al., 2013). Accordingly, *in silico* analysis using String (http://string-db.org) puts this gene in cancer pathways. The other detected rare CNV is a 5q35.3 duplication of 368 kb that harbors the *GRM6*, *ADAMTS2*, *ZNF879* and *ZNF354C* genes. The *GRM6* gene was recently seen hypermethylated in renal cell carcinomas (Arai et al., 2012); *ADAMTS2* acts as a metalloproteinase protein already reported as altered in squamous cell carcinoma (Carinci et al., 2005). In addition, *ADAMTS2* was associated with a 2–3 fold increase of the risk for malignant pleural mesothelioma development (Matullo et al., 2013).

Although we cannot exclude that the occurrence of rare CNVs can be a coincidental finding, our data on rare germline CNVs highlighted genes that, in case of being dosage sensitive, could add to the genetic

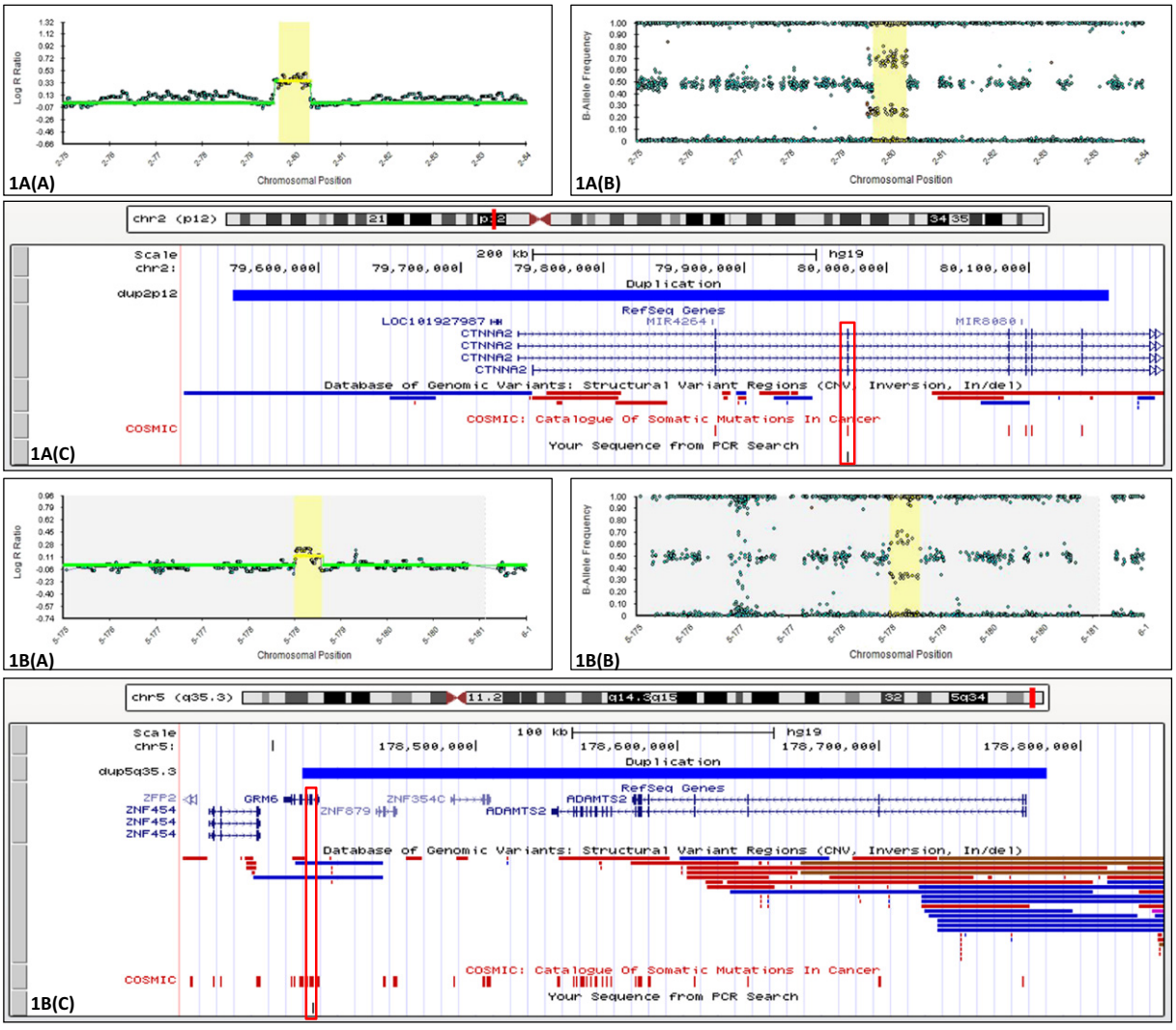

Fig. 1. A: Rare CNV (duplication) affecting a 616 kb genomic region at 2p12 detected by SNP-array in a melanoma/pancreatic cancer patient. (A) Probes above the baseline 0 of the Log-Ratio representing the duplicated segment at 2p12 are highlighted in yellow. (B) Plot of the B-allele frequency exhibiting the pattern of the 2p12 duplication highlighted in yellow. (C) The affected genomic segment at 2p12 seen by the UCSC genome browser: the region encompasses two genes, *CTNNA2* and *MIR4264*, and the sequence used in the qPCR assay is marked by a red box. B: Rare CNV (duplication) affecting a 368 kb genomic region at 5q35.3 detected by SNP-arrays in a melanoma/pancreatic cancer patient. (A) Probes above the baseline 0 of the Log-Ratio representing the 5q35.3 duplication highlighted in yellow. (B) Plot of the B-allele frequency presenting a pattern of the 5q35.3 duplication highlighted in yellow. (C) The affected genomic segment at 5q35.3 seen by the UCSC genome browser: the region encompasses four genes, *GRM6*, *ADAMTS2*, *ZNF879* and *ZNF354C*, and the sequence used in the qPCR assay is marked by a red box.

basis of the cancer predisposition of melanoma associated with a pancreatic neoplasia.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yexmp.2014.06.013.

Disclosure/conflict of interest

The authors have no conflict of interest to declare.

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