Spatiotemporal Heterogeneity Characterizes the Genetic Landscape of Pheochromocytoma and Defines Early Events in Tumorigenesis

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Abstract

Purpose: Pheochromocytoma and paraganglioma (PPGL) patients display heterogeneity in the clinical presentation and underlying genetic cause. The degree of inter- and intratumor genetic heterogeneity has not yet been defined.

Experimental Design: In PPGLs from 94 patients, we analyzed LOH, copy-number variations, and mutation status of SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL, EPAS1, NF1, RET, TMEM127, MAX, and HRAS using high-density SNP array and targeted deep sequencing, respectively. Genetic heterogeneity was determined through (i) bioinformatics analysis of individual samples that estimated absolute purity and ploidy from SNP array data and (ii) comparison of paired tumor samples that allowed reconstruction of phylogenetic trees.

Results: Mutations were found in 61% of the tumors and correlated with specific patterns of somatic copy-number aberrations (SCNA) and degree of nontumoral cell admixture. Intratumor genetic heterogeneity was observed in 74 of 136 samples using absolute bioinformatics estimations and in 22 of 24 patients by comparison of paired samples. In addition, a low genetic concordance was observed between paired primary tumors and distant metastases. This allowed for reconstructing the life history of individual tumors, identifying somatic mutations as well as copy-number loss of 3p and 11p (VHL subgroup), 1p (Cluster 2), and 17q (NF1 subgroup) as early events in PPGL tumorigenesis.

Conclusions: Genomic landscapes of PPGL are specific to mutation subtype and characterized by genetic heterogeneity both within and between tumor lesions of the same patient. Clin Cancer Res; 21(19): 4451–60. © 2015 AACR.

Introduction

Neuroendocrine tumors of the adrenal medulla and paraganglia (pheochromocytoma and paraganglioma: PPGL) display significant interpatient heterogeneity both in terms of clinical symptoms and genotype (1–3). Mutations in a total of 12 genes have been identified as conferring susceptibility to PPGL. In addition, HRAS has been identified with recurrent somatic mutations (1). Stratification according to the mutated gene has revealed distinct clinical and molecular characteristics that form at least three subgroups (4–8). PPGLs in cluster 1a (SDHx mutated) and b (VHL and EPAS1 mutated) are characterized by a predominant norepinephrine secretion and activation of pseudohypoxic signaling pathways. In addition, SDHx tumors have been described to exhibit a hypermethylated signature (5, 9). In comparison, cluster 2 PPGLs (RET, NF1, TMEM127, MAX, and HRAS mutated) exhibit a mixed epinephrine/norepinephrine secretion and increased activity of mitogenic signaling pathways (4, 6). Somatic copy-number alterations (SCNA) have also been described as exhibiting mutation-specific patterns (10). VHL-associated tumors are characterized by deletions of chromosomes 3 and 11, whereas those with RET and NF1 mutations display deletions of the short arm of chromosome 1 (10–12). NF1 tumors show frequent deletions of chromosome 17 and those with mutations in EPAS1 tumor show gains on chromosome 2 (10, 13–15). It has also been shown that malignant PPGLs have more SCNA than benign tumors (16–18).

Characterization of genetic heterogeneity within and between tumors of individual patients has revealed complex mutational landscapes that are variable both in space and time (19–22). Understanding the dynamics and composition of different tumor clones within individual tumors may allow for the identification of driver events in tumorigenesis that could ultimately lead to improved prognostic and predictive biomarkers (23–25). In this study, we sought to investigate spatial and temporal genetic heterogeneity in a large cohort of PPGL by determining their genomic architecture and clonal compositions.

Materials and Methods

Ethics

We have analyzed a total of 138 different samples from 104 tumors obtained from 94 different patients. All biologic specimens were obtained from Uppsala Biobank, Endocrine tumor collection [Ethical approval 00-128/3.15.2000, Local ethical
To detect variants causing aberrant fraction of the tumor (subclonal). In the third round, we aimed at analyzing different locations within the same tumor lesions and overlapping the observed mutation was used to calculate the expected allele frequency of a mutation given that the mutation occurred in a cellular proportion equal to the total sample purity. This was then compared with the observed allele frequency of pathogenic variants, and differences between the expected and observed allele frequencies of more than 10% were considered as statistically significant.

Sample sequencing

Prior to this study, 43 patients had been identified with pathogenic genetic variants (26–29). In this study, we performed three additional rounds of sequencing followed by validation using at least one different sequencing chemistry. The workflow has been outlined in detail in Supplementary Fig. S3. First, all PPGL tumors were subjected to TruSeq Custom Amplicon (Illumina) targeted enrichment and library preparation that had been designed to cover all protein-coding sequences and flanking splice sites of SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL, EPAS1, RET (exons 8, 10–11, and 13–16), NF1, TMEM127, MAX, and H-RAS genes. The generated fragments were then pooled and sequenced twice on an Illumina MiSeq instrument. Seconded, all cases that did not display a pathogenic variant in the first run were subjected to an additional round of targeted enrichment and high-throughput sequencing utilizing the identical procedure. In this second round, we have analyzed different locations within the same tumor in order to identify potential mutations occurring in a fraction of the tumor (subclonal). In the third round, we aimed to detect variants causing aberrant NF1 splicing and performed cDNA sequencing on 13 tumors without mutation but with LOH at NF1 locus (n = 13). In the fourth round, variants predicted as pathogenic were validated with Sanger sequencing and/or high-throughput sequencing of specific PCR amplicons. All methods are described in detail in Supplementary Material.

SNP array analysis

All included samples were analyzed using Illumina Infinium dual-fluorescence single nucleotide polymorphism (SNP) arrays HumanOmnixExpressExome-8v1_A, Omni1-Quad, or Omni2.5-Quad (Illumina). Raw data were processed in Illumina BeadStudio (Illumina; version 7.5 build 2011) that generated measures of total signal intensity (LogR) and relative intensity between the two alleles (BAF) of each given SNP probe. Data were exported and further processed using Nexus copy-number variation 7.5 (Biodiscovery).

Absolute determination of ploidy and purity from SNP array data

Segments with aberrant LogR and/or BAF were generated using an SNP-RANK algorithm and analyzed using a combination of commercial and publicly available softwares and algorithms with the aim of estimating absolute ploidy and clonal fractions. Theory of operations and validation of the study workflow is outlined in detail in Supplementary Material. We utilized a maximization of parsimony approach; when two or more combinations of ploidy and clonal fractions could explain the underlying data, the model prefers the simplest. Each segment was classified through (i) ploidy classification into A, B, AB, AAB, BAA, AABB (defined as simple calls), (ii) determination of cell fraction (0%–100%) from ploidy and B-allele frequency (BAF). Two internal validation steps were performed to calibrate these estimates of absolute ploidy and purity. First, we inspected each sample manually in order to identify sample outliers with more complex ploidy calls. In the second round of validation, we determined allele-specific copy-number status and purity through interpolation to an array-specific relationship between copy number and LogR.

Validation with external resources was performed for tumor fraction (tumor cell fraction) with the use of allele-specific copy-number analysis of tumors (ASCAT) bioinformatics suite version 2.1 (30). Absolute ploidy and clonal fractions of selected samples were validated with Tumor Aberration Prediction Suite (TAPS) as previously described (31).

Clonal predictions

The total tumor purity was calculated from SNP array data as the maximum cell fraction observed for generated segments within each sample. SNP array segments with a difference in cell fraction of more than 10 percentage points from sample purity were considered as occurring in a fraction of the tumor: subclonal classification. In order to determine if mutations occurred in fully-subclonal fashion, we performed integrative analysis of SNP array data. Absolute ploidy and cell fraction from the position overlapping the observed mutation was used to calculate the expected allele frequency of a mutation given that the mutation occurred in a cellular proportion equal to the total sample purity.

This was then compared with the observed allele frequency of pathogenic variants, and differences between the expected and observed allele frequencies of more than 10% were considered as subclonal.
Construction of phylogenetic trees

Phylogenetic trees were drawn using comparison data from paired samples only. Relative SNP array data and pathogenic MiSEQ variants (assigned equal value and together defined as events) were selected. A binary distance matrix was created with each sample as a separate column and each event as a separate row (Supplementary Table S4; ref. 19). Phylogenetic trees were reconstructed using the maximum parsimony method available in the MEGA6 software package (32). In cases where multiple trees were rendered, a bootstrap consensus tree was selected for further analysis. Germline variants and diploid regions were considered as the root. Trees were redrawn in Microsoft Powerpoint (Microsoft Corp) with the lengths of the branches being proportional to the number of events. Events were then classified as ubiquitous (occurring in all samples), shared (occurring in ≥2 samples), or private (occurring in a single sample).

Results

Targeted capture of 12 disease-causing genes followed by massive parallel sequencing resulted in a mean read coverage of 200 (Supplementary Table S1). Mutually exclusive pathogenic mutations in SDHB (n = 2), VHL (n = 15), EPAS1 (n = 2), NF1 (n = 17), RET (n = 15), and H-RAS (n = 6) genes were present in 61% (57/94) of the tumors (sensitivity 98.7% compared with Sanger sequencing; Fig. 1 and Supplementary Fig. S3A and S3B). Tumors without pathogenic mutation were re-sampled and subjected to another round of sequencing (mean read coverage 282). No further disease-causing variants were found in this round of sequencing.

Figure 1.
Genomic landscape of pheochromocytoma. Heatmap of SCNA in primary tumors clustered accordingly to mutated gene. Each row represents a unique primary tumor from a single patient. From the left, indication of mutated gene, presence of metastasis, heatmap representing losses and gains as well as allelic imbalances with normal copy number. The total tumor cell fraction of each sample is plotted as a black line with the red lines representing mean tumor cell fraction for each mutation subgroup. Cnn, copy number neutral.

Figure 2.
Descriptive numbers of SCNA classification in pheochromocytoma. A, distribution of SCNA as copy-number gain and loss as well as in copy-number neutral allelic imbalances. B, relationship between SCNA and the fraction of the genome with SCNA.
A total of 1,159 SCNA were detected by SNP array in 136 tumor samples (Supplementary Table S3). The median number of SCNA per sample was 7 (range, 0–21), resulting in a median proportion of genome with SCNA of 0.16 (range, 0%–80%; Fig. 2B). Hyperploid DNA content (ploidy >2.5) was observed in tumors from 2 patients. Median tumor purity was estimated from SNP array data to 74% (range, 17%–100%) consistent with values acquired from validation with ASCAT (Pearson correlation \( R = 0.830 \); Supplementary Fig. S6). Histologic examination revealed slightly lower estimates of tumor purity with a median value of 67% (range, 40%–90%; Pearson correlation \( R = 0.658 \); Supplementary Fig. S7).

Intra- and intertumor heterogeneity in PPGL

The degree of genetic heterogeneity was estimated (i) in all individual samples and (ii) between paired multiregional samples using bioinformatics analyses (Fig. 3A). Consistency between data generated from next-generation sequencing and SNP array was confirmed by comparing the allele frequencies of known SNPs as well as germline mutations to median BAF of the overlapping segment (Pearson correlation \( R = 0.945 \); Supplementary Fig. S9A). Expected mutation allele frequencies, assuming they occur in 100% of tumor cells, were calculated from SNP array data and compared with the observed allele frequency. These results suggest that somatic mutations occurred in all tumor cells \((R = 0.965;\) Fig. 3B). This was different to SCNA that occurred as subclonal events in 74 of 136 tumor samples. The median fraction of SCNA classified as subclonal per sample was 8% (range, 0%–92%; Fig. 3C).

A total of 21 primary tumors were subjected to multiregional sampling that revealed discrepant SCNA patterns in 18 of the cases (Fig. 4A and B). In primary tumors from patients 55, 57, and 87, we could not detect differences between samples from different regions. Tumors from patients without metastatic disease \((n = 18)\) were characterized by a low degree of genetic heterogeneity with a median genetic divergence of one event \((\text{range, 0–6; Fig. 4A})\). In contrast, the genetic landscapes of patients with distant metastases showed more pronounced intratumor heterogeneity. In the primary tumors of 3 patients with distant metastases, there were 5, 26, and 17 discrepant events in patients 25, 70, and 78, respectively \((\text{Fig. 4B})\). Including SCNA and mutations discovered in all tumors from patients with distant metastases, the proportions of ubiquitous events were 0.18, 0.32, and 0.14 and for patients 25, 34, and 71, respectively. The subsequent evolution of distant metastatic lesions displayed diverse patterns with branched development of different tumor cell clones observed in patient 34. In comparison with distant metastases, local recurrences with metastatic invasion were more closely related to the primary tumor and exhibited a low degree of intratumor heterogeneity.

Genetic subgroups define genetic landscapes in PPGL

When grouped according to mutated gene, tumors revealed distinct differences in the SCNA patterns dependent on the detected mutation \((\text{Fig. 1})\). Difference in the number of SCNA in the primary tumors was also correlated to mutated genes and carrier status \((\text{Kruskal–Wallis test } P = 0.003 \text{ and Mann–Whitney } U \text{ test } P = 0.023; \text{ Fig. 5A, Table 1})\). Tumors from patients with germline VHL mutations had the least number of SCNA, whereas those with \(NFI\) mutation or somatic carrier status independent of mutation subtype had more frequent SCNA.

Differences in tumor purity were also observed between mutation subgroups. Proangiogenic PPGL defined as known VHL mutation or relatively high VEGFA gene expression, including those with unknown mutation status, had pronounced contamination of nontumor cells compared with other cases \((\text{Kruskal–Wallis test } P = 0.003 \text{ and Mann–Whitney } U \text{ test } P = <0.001; \text{ Fig. 5B–D and Supplementary Tables S2 and S3})\). These correlations were independent of germline/somatic mutation status.

In order to generate chronological classification, we separated tumors according to mutation and annotated events pursuant to their clonal status and their position in the phylogenetic tree. Events were considered as occurring early in the tumor development if classified as fully clonal and located at the trunk of the phylogenetic tree. Those that were either classified as subclonal or were located in the phylogenetic branches were considered as occurring late in tumor development. Biallelic inactivation of the VHL locus as well as copy-number loss of 11p was identified as both fully clonal and trunclal events in all VHL-mutated PPGLs. Similarly, loss of 1p was observed among all cluster 2 as well as SDHB-mutated cases \((\text{Fig. 3D})\). Loss of chromosome 3q was identified in all HRAS and RET-mutated PPGLs and in 12 of 17 of those with \(NFI\) mutation. Additional mutation-specific facultative events were observed \((\text{Fig. 3D})\): all but one \(NFI\)-mutated tumors had biallelic loss of the \(NFI\) gene and all \(RET\) and HRAS mutations were classified as ubiquitous. In addition, \(RET\)-mutated cases had frequent loss at chromosome 21 \((9/13 \text{ cases})\), \(HRAS\)-

Phenotype correlations

Detailed descriptive statistics and test results are present in Supplementary Tables S2 and S3. Comparison of benign and malignant PPGLs revealed that benign tumors had fewer SCNA per tumor lesion: median, six events in nonmetastatic versus 16 events in metastatic cases \((\text{Mann–Whitney } U \text{ test } P < 0.001; \text{ Fig. 5G–I; Supplementary Table S3})\). Fraction of the genome with SCNA was lower among benign \((\text{median, 0.14; range, 0–0.62})\) compared with metastatic tumors \((\text{median, 0.26; range, 0.05–0.8})\). Mann–Whitney \( U \text{ test } P < 0.001; \text{ Fig. 5G})\). These differences were not statistically significant in primary tumors, precluding identification of a cut-off separating benign from metastatic primary tumors \((\text{Supplementary Table S2})\). Pheochromocytoma primary tumors showed a strong tendency to have more SCNA than paraganglioma \((\text{Mann–Whitney } U \text{ test } P = 0.062)\). Large primary tumors \((>60 \text{ mm})\) and those resected from older patients \((>50 \text{ years at diagnosis})\) were characterized by higher number of SCNA \((\text{Mann–Whitney } U \text{ test } P = 0.004 \text{ and 0.023; Fig. 5E and F})\). Larger tumors had a higher tumor purity \((\text{Mann–Whitney } U \text{ test } P = 0.018)\) compared with smaller tumors. Males had a trend to accumulate more SCNA than females \((\text{Mann–Whitney } U \text{ test } P = 0.053)\).
Figure 3.
Clonal status of mutations and structural events. A, outline of the two methods used in determining genetic heterogeneity. By estimating the absolute purity and ploidy of each event, the degree of genetic heterogeneity within each sample was performed. Genetic heterogeneity was also determined from paired samples that allowed construction of phylogenetic trees. B, from SNP array data, the allele frequency of each mutation was determined assuming it occurred in all cells within that tumor. This was compared with the observed allele frequency of each mutation. Green dots represent pathogenic mutations classified as present in constitutional DNA, and blue dots represent mutations classified as present exclusively in tumor DNA. C, classification of copy-number gain versus loss and copy-number neutral allelic imbalances according to clonal status for 138 samples from 104 tumors from 94 patients. D, frequency of mutations (first columns) as well as five most commonly associated loci with copy-number losses (including copy-number neutral LOH) and gains in the same tumors presented for HRAS, NF1, RET, and VHL mutation subgroups. Data were further segregated into either early status (phylogenetic trunk and fully clonal) or late status (phylogenetic branch and/or subclonal).
within tumor lesions and between tumor lesions from the same patient. These results defined the chronological order of mutations and SCNA that may ultimately improve our understanding of PPGL tumor biology and the progress of the disease.

Genome-wide copy-number analysis revealed that PPGL tumors are characterized by a relatively low degree of chromosomal instability compared with solid tumors of epithelial origin (33, 34). Among patients with relatively high aneuploidy, we could identify two subgroups characterized by large and small SCNA, which could reflect differences in the mechanisms underlying genomic instability.

In order to generate predictions of absolute ploidy and tumor cell proportions from the SNP array data of these relatively stable genomes, we chose a maximum parsimony model that was validated against established bioinformatics tools: ASCAT and TAPS as well as against histopathologic examination. Technical cross-validation of the generated BAF calls and allele frequencies was also performed against targeted deep sequencing. Although the conducted validation steps indicate robustness of our observations, utilizing a maximum of parsimony approach has its limitations. Biology may behave differently than the simplest theorization (35), and in order to reduce the proportion of miscalled segments, we calibrated each observation to empirical data obtained from the infinium array (36). Complementary investigation using single-cell or in situ techniques could decrease the risk of false estimations and deduce the genetic overlap in polyclonal samples (21, 37).

We demonstrated spatial genetic heterogeneity in a majority of PPGL tumors. This was restricted to SCNA while mutations in the targeted genes occurred exclusively in a ubiquitous fashion. The degree of intratumor heterogeneity was relatively small compared with malignant carcinomas (19, 21, 38). Subclonal SCNA were evident both from bioinformatics analysis of individual samples and by comparison of paired samples from identical tumors. A higher degree of genetic intratumor heterogeneity was observed by comparison of paired samples than that observed from bioinformatic analysis of single samples. This could reflect that heterogeneity in PPGL increases with spatial distance. However, the bioinformatics workflow may have underestimated the proportion of subclonal SCNA.

PPGL with evidence of metastasis tended to have higher degree of subclonal events and aneuploidy compared with the nonmetastatic lesions, consistent with previous studies (16–18). However, evaluating genomic instability and genetic heterogeneity as prognostic biomarkers in primary tumors failed to determine cutoffs for risk of metastatic disease. We proceeded with qualitative observations that identified primary tumors from patients with local metastatic recurrences as...
having pronounced chromosomal instability with a relatively low degree of intra- and intertumor heterogeneity. This was in contrast with primary tumors from patients with distant metastatic lesions that were characterized by a lower degree of aneuploidy but having pronounced inter- and intra-tumor genetic heterogeneity, in line with previous observations (39, 40).

Robust evidence supports differences in transcriptome and methylome of PPGL tumors with different mutations (4, 9). In this study, we detected pathogenic mutations in 58 cases that enabled us to provide further data to an SCNA landscape that is similarly dependent on and unique to the specific mutated gene. By stratifying cases according to mutation and analyzing absolute ploidy, tumor cell fractions as well as position within the phylogenetic tree allowed temporal categorization and identification of events that occurred early in tumorigenesis (22). Loss of chromosome 1p (cluster 2 PPGL), oncogenic mutations (HRAS and RET), biallelic inactivation of 17q (NF1-mutated cases) as well as biallelic inactivation of VHL and loss of 11p (VHL-mutated cases) were obligate events occurring early in PPGL development (11, 16, 41). Differences in the chronological order of somatic driver mutations and obligate SCNA were not detectable, suggesting that both are needed for initial clonal expansion that occurred from a limited population of tumor cells. This mutation-specific SCNA landscape supports a model of parallel evolution during the early stages of PPGL tumorigenesis where mutation subtype predicts a specific path from precursor cell to tumor development (42, 43). We hypothesize that during the continuous expansion of the tumor cell population, stochastic SCNA accumulate, manifesting as either SCNA with low cohort frequency and/or having subclonal status. Furthermore, PPGL may acquire malignant transformation through these random SCNA, supported by the clear divergence between the clones detected within primary and metastatic tumors from patients 25 and 71. These results suggest that the malignant clone might not be captured in PPGL primary tumors through a single-biopsy approach. Thus, genetic intratumor heterogeneity could potentially obstruct the identification of markers specific to malignant PPGL. Instead the ubiquitous status of driver mutations emphasizes their potential to serve as prognostic and predictive markers as well as identifiers of druggable targets in PPGL. The ultimate implications of genetic heterogeneity in PPGL need to be clarified in future studies using genome-wide single-nucleotide resolution (44).
The fraction of tumor cells within each sample was assessed from SNP array data and histopathologic examination. As previously described in the literature (34), histopathologic observation tended to overestimate the proportion of tumor cells, especially for samples that had a high proportion of infiltrating stromal cells. We found that tumors with pseudohypoxic characteristics determined by either mutation subtype or gene expression had higher proportions of non-tumor cells. Although this could reflect how samples were surgically resected and macrodissected at our center, infiltrating stromal cells within pseudohypoxic tumors (45) could have been a contributing underlying factor behind this observation. This finding could ultimately have implication for investigating and interpreting transcriptome and methylome data in PPGL.

In summary, our analysis identified mutation-specific copy-number aberrations and clonal architectures in PPGL with genetic heterogeneity on all levels: between patients, between different tumor lesions of the individual patient, and within specific tumor lesions.

Table 1. Cohort information

<table>
<thead>
<tr>
<th>Patients, n</th>
<th>Tumors, n</th>
<th>SNP array samples, n</th>
<th>Deep sequencing samples, n</th>
<th>Paired analysis, n</th>
<th>Age, years median (range)</th>
<th>Tumor size, median (range)</th>
<th>Gender, n female/male</th>
<th>Syndromic patients, n</th>
<th>Type, n PCC/PGL</th>
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<td>93</td>
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<td>55 (5–170)</td>
<td>56/37</td>
<td>20</td>
<td>82/11</td>
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NOTE: Details of the investigated cohort. Paired analysis was defined as when both SNP array and targeted deep sequencing were applied to the identical batch of gDNA.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Crona, P. Stålberg, P. Björklund

Development of methodology: J. Crona, S. Backman, P. Björklund

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Stålberg, P. Hellman, P. Björklund

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Crona, S. Backman, M. Mayrhofer, P. Stålberg, A. Isaksson, P. Hellman, P. Björklund

Writing, review, and/or revision of the manuscript: J. Crona, S. Backman, R. Maharjan, M. Mayrhofer, P. Stålberg, A. Isaksson, P. Hellman, P. Björklund

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Crona, P. Hellman, P. Björklund

Study supervision: P. Stålberg, P. Hellman, P. Björklund

Other (contributed in lab experiment/studies): R. Maharjan

References


