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Target-Enriched Next-Generation Sequencing Reveals Differences between Primary and Secondary Ovarian Tumors in Formalin-Fixed, Paraffin-Embedded Tissue

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Differentiating primary endometrioid or mucinous ovarian tumors from secondary ovarian tumors can be challenging. We compared somatic mutation profiles of primary and secondary ovarian cancers to investigate if these profiles can help diagnose ovarian tumors. Cancer-related genes ($n = 115$) were screened by target-enriched next-generation sequencing in formalin-fixed, paraffin-embedded tumor tissue from 43 primary endometrioid and mucinous ovarian carcinomas and 28 proven colorectal cancer metastases to the ovary. Results were validated by high-resolution melting curve analysis and Sanger sequencing. *TP53*, *NOTCH1*, *PIK3CA*, and *FAT4* versus *APC*, *TP53*, *KRAS*, and *FAT4* mutations were the most common in the primary ovarian tumors and ovarian colorectal cancer metastases, respectively. An inactivating *APC* mutation was found in 4.7% of primary ovarian tumors (2 of 43; 95% CI, 1.6%—10.9%). In contrast, inactivating *APC* mutations were identified in 71% of colorectal cancer metastases (20 of 28; 95% CI, 55%—88%) ($P < 0.001$; sensitivity: 71.4%, 95% CI, 51.1%—86.0%; specificity: 95.4%, 95% CI, 82.9%—99.1%). Loss of heterozygosity and *APC* promoter hypermethylation did not differ significantly between the primary and secondary ovarian tumors. *NOTCH1* mutations were observed specifically in primary ovarian tumors, although at a low frequency, but not in metastases (6 of 41; 14.6%; 95% CI, 3.8%—25.4%). *APC* mutation analysis can be used to differentiate primary endometrioid and mucinous ovarian tumors from colorectal cancer metastases to the ovary. *(J Mol Diagn 2015, 17: 193—200; http://dx.doi.org/10.1016/j.jmoldx.2014.10.006)*

Ovarian tumors can be subdivided into primary (85%) and secondary (metastases; 15%) tumors. For therapeutic and prognostic reasons, it is important to be able to distinguish between these two categories.[1] Roughly one-half of the primary tumors that give rise to ovarian metastases are located in the gastrointestinal tract.[2,3] Although these are mostly colorectal cancers (CRCs), tumors in the appendix, stomach, or pancreas can also metastasize to the ovary. These metastasizing primary gastrointestinal tract tumors are mostly conventional adenocarcinomas and are often easily recognized as such on the basis of histology and immunohistochemical stainings. However, some of the primary ovarian malignancies such as endometrioid and mucinous adenocarcinomas can show extensive histologic and immunohistochemical similarities to primary gastrointestinal tract tumors, making a final diagnosis difficult.[4,5] Other frequently observed subtypes, such as serous papillary carcinomas of the ovary, do not pose such problems.

Size, bilaterality, and immunohistochemical staining of ovarian lesions can be used to differentiate between primary and secondary tumors.[6–11] Nevertheless, the tools that are currently available cannot always discriminate adequately and present a risk of equivocal characterization of ovarian

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tumors. Therefore, finding molecular information that further characterizes some of these ovarian lesions would be helpful in daily practice. The COSMIC (Catalogue of Somatic Mutations in Cancer) database, currently the foremost database of (somatic) mutation profiles,[12] shows that similar cancer-driving mutations can be found in several types of cancer, although these mutations are often observed at different frequencies.[13,14]

In this study, we focused on ovarian metastases of CRC. On the basis of mutation profiles described in the literature and the COSMIC database, *APC* appears to be the best candidate for discriminating between primary tumors versus metastases of CRC.[4,15,16]

One hundred fifteen cancer-related genes (including *APC* and *CTNNB1*) were analyzed in the formalin-fixed, paraffin-embedded (FFPE) material, which is used in diagnostics on a daily basis, by target enriched next-generation sequencing (NGS).[17] In addition, methylation and loss of heterozygosity (LOH) of *APC* were investigated. In this study, we show that the detection of inactivating *APC* mutations can help to distinguish primary from secondary ovarian tumors.

Materials and Methods

Tissue Samples

Ovarian metastases of CRC were obtained from the archives of the Leiden University Medical Center Pathology Department (period, 1985 to 2010; $n = 10$) and from PALGA (the nationwide Dutch network and registry of histopathology and cytopathology; $n = 18$).[18] FFPE tissue was available for all 28 ovarian metastases of CRC, fresh frozen for 6 metastases. Next, 35 endometrioid and 7 mucinous primary ovarian cancers were selected, because these subtypes can pose diagnostic problems on the basis of their histologic similarity with CRC metastases to the ovary. One case showed a mixed type histology that consisted of an endometrioid tumor with clear cell elements. Both frozen and FFPE tissues were available for all of the primary ovarian tumors.

Medical Consent

The present study falls under approval by the Medical Ethical Committee of the Leiden University Medical Center (protocol P01-019). Informed consent was obtained according to protocols approved by the Leiden University Medical Center Medical Ethical Committee (02-2004). The patient samples were handled according to the medical ethics guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (*http://www.federa.org/codes-conduct*, last accessed January 2013).

Tissue (Micro)-Dissection and DNA Extraction

On basis of pathologic examination of hematoxylin and eosin–stained slides tumor tissue that contained >50% tumor cells was selected from FFPE material. Tumor tissue was taken by using a 0.6- to 2.0-mm tissue cylinder, depending on the tumor volume (Beecher Instruments, Sun Prairie, WI). In case tumor fields were too small for punching, microdissection was performed as follows: 5 to 10 × 10 μmol/L slides were cut and stained with hematoxylin only. Eosin staining was omitted to preserve the integrity of the DNA. After staining, slides were visualized with an inverted microscope and were microdissected with a sharp-pointed knife. When frozen tissue was used, tumor enrichment was achieved by removing nontumorous tissue as much as possible after frozen section analysis. DNA was isolated with the Nucleospin Tissue kit (Bioke, Leiden, the Netherlands). After isolation the DNA was dissolved in 50 to 100 μL of water. The concentration of the DNA ranged from 10 to 200 ng/μL for microdissected tumors with dispersed tumor fields and solid tumors, respectively.

Targeted Sequencing in FFPE Tissue

To screen 115 genes, a HaloPlex custom-made target enrichment kit was designed (Agilent Technologies, Santa Clara, CA). Genes were selected on the basis of the literature (Supplemental Table S1).[19,20] Library preparation was performed, with 250 ng as input, according to the standard protocol (Agilent Technologies). After equimolar pooling, the samples were sequenced on an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA). An average coverage of 359× was achieved (range, 15× to 1972×; median, 355×).

Data Analysis

Adaptors, barcodes, and enzyme footprints were removed from the sequenced reads by using SureCall software version 1.1.0.15 (Agilent Technologies), after which the reads were aligned with Burrows-Wheeler aligner.[21] The Genome Analysis Toolkit (Broad Institute, Cambridge, MA) was used for realignment around indels and base quality recalibration.[22] Removal of duplicates is not necessary in HaloPlex hybridization-extension experiments that are used to capture the target DNA regions. Single nucleotide polymorphism and indel calling were performed with varScan 2 package version 2.3.6 by using the following arguments: minimum read depth = 8, minimum number of reads with the alternative allele = 2, minimum base quality = 20, and minimum variant allele frequency = 0.10. Details on total reads and coverage are given in Supplemental Table S2.

Variants were functionally annotated with ANNOVAR.[23,24] To remove false positives, three strategies were used. First, the variants with supported reads in only one direction in which the wild-type allele had more than two reads in both directions were removed. Second, the variants with a frequency >90% in an extended cohort of 102 patients were considered to be false variants and were removed. Third, the variants in known duplicated genomic regions were removed from downstream analysis. Matched normal tissue