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Ovarian Cancer Biomarker Discovery Based on Genomic Approaches

REVIEW

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Ovarian cancer presents at an advanced stage in more than 75% of patients. Early detection has great promise to improve clinical outcomes. Although the advancing proteomic technologies led to the discovery of numerous ovarian cancer biomarkers, no screening method has been recommended for early detection of ovarian cancer. Complexity and heterogeneity of ovarian carcinogenesis is a major obstacle to discover biomarkers. As cancer arises due to accumulation of genetic change, understanding the close connection between genetic changes and ovarian carcinogenesis would provide the opportunity to find novel gene-level ovarian cancer biomarkers. In this review, we summarize the various gene-based biomarkers by genomic technologies, including inherited gene mutations, epigenetic changes, and differential gene expression. In addition, we suggest the strategy to discover novel gene-based biomarkers with recently introduced next generation sequencing. **(J Cancer Prev 2013;18:298-312)**

Key Words: Cancer biomarker, Ovarian cancer, Genomic technologies, Genes, Early detection

INTRODUCTION

Ovarian cancer is the leading cause of mortality from female reproductive cancer. Although it is the ninth most common cancer, it is among the five leading causes of cancer death in women.[1] No definite symptoms related with early-stage disease and no effective screening methods make its early detection difficult, which results in about two-third patients with advanced-stage ovarian cancer at the diagnosis.[2] When we consider that 5-year survival rate is up to 90% in patients with early-stage ovarian cancer while it is less than 20% in those with advanced-stage disease, the development of effective screening tests and their applications in clinical setting are very important to improve the prognosis of ovarian cancer by early detection.[3]

Cancers are caused by the accumulation of genetic damages, but the genetic mutations and pathways for early ovarian carcinogenesis are largely unknown. Considering the close relationship between genetic alterations and ovarian carcinogenesis, the research on gene level may be expected to provide novel ovarian cancer biomarkers.[4] With application of second-generation DNA sequencing technologies, it has become feasible to fully sequence whole genome more fast and less cost than traditional ones. The focus of this review is on ovarian cancer with challenges of its early detection and how recent development of genomic technologies could contribute to overcoming these challenges.

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OVARIAN CANCER AND CHALLENGES IN ITS EARLY DETECTION

Ovarian cancer is relatively a rare disease with an incidence of less than 40 per 100,000 per year.[1] Thus, the ideal screening method has high specificity to avoid unnecessary surgery-related complications, and high positive predictive value to detect early-stage ovarian cancer which can be treated by optimal debulking surgery with adjuvant chemotherapy. Considering the low incidence of ovarian cancer, most of researchers agree that a screening test should satisfy sensitivity of >75%, specificity of > 99.6% and positive predictive value of >10%.[5-7]

Several biomarkers have been developed as feasible tools for the early detection and follow-up of ovarian cancer. In particular, CA-125, the most widely used serum biomarker for ovarian cancer, has a value for monitoring tumor response and disease recurrence after treatment.[5] Approximately 80% of patients with advanced-stage ovarian cancer show elevated serum CA-125 levels; more than 50% of those with early-stage disease demonstrate normal values.[8] Moreover, it has high false-positive rate of 30%, showing elevated levels in benign conditions including pregnancy, pelvic inflammatory disease and endometriosis.[9]

To overcome these limitations of serum CA-125 levels as a screening test, numerous efforts have been made by combining with imaging modalities or other biomarkers. In particular, CA-125 followed by transvaginal ultrasonography showed encouraging results with sensitivity (SN) of 89.5%, specificity (SP) of 99.8% and positive predictive value (PPV) of 35.1% for detecting early-stage ovarian cancer in the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) study.[10] However, the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening trial has shown that the combination of CA-125 with transvaginal sonography failed to reduce ovarian cancer mortality in postmenopausal women when compared with usual care (RR, 1.18; 95% CI, 0.82-1.71), and relevant complications from surgery after the combined screening test were problematic because of high false-positive results.[11]

CURRENT STATUS OF SCREENING OF OVARIAN CANCER

Up to now, only two biomarkers, CA-125 and HE4, have been approved by FDA for monitoring disease recurrence and therapeutic response, not for screening. Numerous efforts have been made to evaluate the combination of multiple serum biomarkers. To date, more than 30 serum markers have been evaluated alone and in combination with CA-125.

The FDA approved OVA1™ test for triage patients with ovarian cancer for surgery by gynecologic oncologists. Immunoassays for CA-125 and four of biomarkers (TT, Apo-A1, β2M, and transferrin) constitute the OVA1™ test. A prospective, multi-institutional trial was conducted to evaluate the performance of the College guidelines with OVA1™ instead of CA-125 alone.[12] The College guideline composed of CA-125, presence of ascites and evidence of metastasis, and family history has been recommended for preoperative consultation to gynecologic oncologist.[13] The study enrolled 590 women with ovarian mass verified by an imaging study. Among them, 516 were evaluable with 161 malignancies. The college guideline with OVA1™ replacing CA-125 increased the sensitivity (77→94) while decreasing specificity (68→35) and positive predictive value (52→40). Sensitivity in the study was remarkable, considering under-estimation by the study design which discriminates cancer from benign ovarian tumor. However, due to decreased specificity and high false positive rate, further study is needed whether guidelines in the study could be applied as a screening test for asymptomatic population.

OvaCheck™ test has been developed according to principles of proteomic technology, using the 11 analytes. Electrospray ionization method was used to detect the proteomic patterns of filtered samples. Although OvaCheck™ has generated substantial publicity, we could not find any peer-reviewed report to analyze. Society of Gynecologic Oncologists issued that more research is needed to validate the test's effectiveness before applying to the public.[14] The test combining leptin, prolactin, OPN, IGF-II and MIF with CA-125 has been developed, which is called as OvaSure™.[15] It was validated with a blinded cohort, and in turn, showed high accuracy with SN of 95.3% and SP of 99.4%. In

particular, SN of the test was 91.6% for early-stage ovarian cancer. However, the diagnostic accuracy for detection of early-stage ovarian cancer has a possibility of over-estimation because only 13 patients with stage I disease were included among a total of 156 with ovarian cancer. In addition, the study was criticized due to having statistical errors. Although only the training set is used to select model and only the test set is used to evaluate the accuracy, the performance of the test was evaluated from combined data.[16]

THE ORIGIN AND PATHOGENESIS OF OVARIAN CANCER

Since proteomics has been introduced in the cancer research, it has been expected that many novel biomarkers would be developed during more than 10 years. However, most of biomarkers have shown disappointing results through clinical trials. As a result, none of the proteins turned out to be better than CA-125 alone.[17] Complexity and heterogeneity of ovarian cancer is a major obstacle to discover novel biomarker. Considering these limitations, it will not be effective that all types of ovarian cancer could be detected with single biomarker. The recent studies have demonstrated that epithelial ovarian cancer is not a single disease but is composed of a diverse group of tumors. Based on distinctive morphologic and molecular genetic features, Shih et al., have proposed a dualistic model that classify various types of ovarian cancer into two groups designed type I and type II.[18] Type I tumors are clinically indolent and usually present with low grade carcinoma, including low-grade serous, low-grade endometrioid, clear cell and mucinous carcinoma. Type II tumors are highly aggressive and almost present in advanced-stage disease, including high-grade serous, high-grade endometrioid and undifferentiated carcinoma.

There are several distinctive genetic mutations that distinguish type I and type II tumors. Type I tumors are genetically more stable than type II tumors and present specific mutations according to different histologic types. BRAF and KRAS mutations, both of which are associated with oncogenic MAPK signaling pathway,[19] are the most significant molecular genetic alterations among type I tumors. Mutations in either at codon 599 of BRAF or codon 12 and 13 of KRAS were found in 68% of low grade invasive serous carcinoma and 61% of serous borderline tumors, but rarely found in high-grade serous carcinoma.[20] KRAS mutations also occurred in 60% of mucinous, 5-16% of clear cell and 4-5% of low grade endometrioid tumor.[18] PTEN is mutated in 21% of type I endometrioid ovarian tumors, rising to 46% in those tumors with 10q23 loss of heterozygosity.[21] WNT and TGF-β signaling pathways are also deregulated in type I carcinogenesis, showing mutations of β-catenin was found in approximately one-third of cases in type I endometrioid cancer and TGF-β mutations occurred in 66% of clear-cell carcinoma.[22,23]

In contrast to type I serous carcinoma in which p53 mutations are rare, mutations in p53 are frequently found in type II tumors. High-grade serous carcinoma, the prototype of type II malignancy, demonstrates high percentage of P53 (50-80%),[24-28] and also presents amplification of HER2/neu (20-67%),[29] and mutation of AKT2 (12-18%).[30,31] These type II tumors are genetically unstable and high-grade mitotic index, showing evolve rapidly and are associated with an early and more aggressive metastatic potential.[32]

The origin of ovarian cancer is still unclear. It is widely believed that various epithelial ovarian tumors arise in the celomic epithelium that covers the ovarian surface and subsequent metaplastic changes lead to the development of the different cell types. However, several evidences oppose this hypothesis.[14] The three most common histological subtypes of epithelial ovarian cancer, referred to as serous, endometrioid, and mucinous, are morphologically resemble the epithelia of the fallopian tube, endometrium, and endocervix, repectively. Moreover, Normal ovary has no components that resemble any of these organs. Therefore, alternative hypothesis suggest that tumors with a müllerian phenotype (serous, endometrioid, mucinous, and clear cell) are derived from embryological identical structure called müllerian tissue, not from mesothelium.[14] One of the potential sites of origin within müllerian duct were the fimbriated end of the fallopian tube. Numerous efforts to find the precursor lesion for ovarian cancer in ovaries have failed, but dysplastic lesions were found in fallopian tubes from women who predisposed to develo-

ping ovarian carcinoma.[33] These results suggest the potential precursor lesion of fallopian tube for ovarian cancer. In addition, serous tubal intraepithelial carcinoma (STIC) is identified in 63% of primary peritoneal carcinoma and 49% of high-grade serous carcinoma.[34] This suggests further evidence supporting the fallopian tube as a source of high-grade serous carcinoma as well as hereditary ovarian cancer. A genetic link between STIC and high-grade serous carcinoma demonstrate STIC as part of cancer spectrum associated with ovarian cancer.[34] STIC and ovarian carcinoma contained identical p53 mutations. Further evidence comes from a gene profiling study showing that gene expression profile of high-grade serous carcinoma is more closely associated with the fallopian tube rather than ovarian surface epithelium.[35] Although several evidences supports for fallopian tube as the origin of ovarian cancer, further studies and more samples are needed to approve STIC as a precursor lesion for high grade serous carcinoma. Efforts to understand the pathogenesis of ovarian cancer should be performed to discover novel ovarian cancer biomarkers.

GENE-BASED APPROACHES TO DIAGNOSIS OF OVARIAN CANCER

Since sequencing the entire human genome was achieved for the first time in 2001, genome technology has led to improvement in the diagnosis of cancer and the selection of cancer treatment. These advances in technology are important for widening our understandings for the pathogenesis of ovarian cancer, which is fundamentally a disease of genome. Considering close relationship between genetic mutations and ovarian tumorigenesis, it is certain that research on gene level would provide novel ovarian cancer biomarker. We categorized to several types of gene-based ovarian cancer biomarker: Inherited gene mutations, epigenetic changes, gene expression, and whole genomic sequencing (Table 1).

1. Inherited gene mutations

It is currently accepted that at least 10% of all epithelial ovarian cancers are hereditary, with mutations in the BRCA1 and BRCA2 genes accounting for approximately 90% of the cases and most of remaining 10% caused by HNPCC.[36-38] Patients with a family history of ovarian cancer are categorized into 3 main groups: (1) "site-specific" ovarian cancer, (2) breast and ovarian cancer syndrome, and (3) hereditary nonpolyposis colorectal cancer (HNPCC).[39] The first 2 groups are related with germline mutations in BRCA1 and BRCA2 gene, and HNPCC syndrome is caused by the germline mutations of the DNA mismatch repair genes, mainly hMLH1 and hMSH2.

Generally, 1 in 280 women carries a germ line BRCA mutation. However, very high frequency of carrier was found in the specific races, such as Ashkenazic Jewish population up to 1/40.[36] For the suspected persons who

Table 1. Potential gene-based biomarkers for ovarian cancer

Types of markers	Strategies	Markers
Inherited gene mutations	Mutations	BRCA1 and BRCA2[39]
		MSH2, MLH1, MSH6, PMS2[38]
		RAD51C, RAD51D, BRIP1, BARD1, CHEK2, MPE11A, NBN, PALB2, RAD50, TP53[43]
Epigenetic changes	Hypermethylation	BRCA1, RASSF1A, APC, p14ARF, p16INK4a, and DAPKinase[59]
		ARMCH1, ICAM4, LOC134466, PEG3, PYCARD and SGNE1[60]
	miRNAs	miR-200a, miR-141, miR-199a, miR-140, miR-145, and miR 125b1[63]
		miR-182[66]
		miR-21, miR-92, miR-93, miR-126, miR-29a, miR-155, miR-127, and miR-99b[68]
Gene expression	Microarray	CA125, osteopontin, kallikrein 10, secretory leukoprostease inhibitor, and matrix metalloproteinase-7[70]
		FOL3, survivin, MCM3, E2Fs, VTCN1, SYNE1, AKAP14, KNDC1, and DLEC1[71]
		ovarian cancer prognostic profile (115 gene signature)[71]
Whole genomic or exome sequencing	Second-generation sequencing	A deletion of TP53[90]
		Frame shift mutations in BRIP1[91]
		TP53, BRCA1, BRCA2, NF1, RB1, FAT3, CSMD3, GABRA6, and CDK12[92]

have the high possibility of hereditary ovarian cancer, genetic testing for BRCA gene should be performed after genetic counseling by cancer genetics professionals. BRCA1 and BRCA2 genes are large tumor suppressor gene located on chromosome 17q21 and 13q12-13, respectively.[40,41] Previous studies showed that both BRCA proteins participate in multiple functions, such as DNA repair, transcriptional regulation of gene expression, and cell cycle.[42] More than 250 mutations may occur in both BRCA genes. Main mutations are frameshift or nonsense variety, accounting for 80% of BRCA gene mutations.[39] These mutations affect the creation of stop codons and protein truncations. In U.S., Myriad Genetics has gene patents and therefore only company to offer clinical testing for BRCA1 and BRCA2.[43] The company offers standard test which includes sequencing of all the coding exons, intron-exon boundaries, and five common gene rearrangements. However, some other rearrangements, which account for 8-15% of all BRCA1/2 mutations, are not identified by standard sequencing.[44-48] Myriad Genetics provide a separate test for this at a cost of 650$. Because additional test is not covered by Medicare and many private insurance companies, the majority of women who tested for BRCA gene have chances for incomplete mutation evaluation and false-negative results.[43] This point should be considered when performing BRCA test to patients. Risk assessment and treatment plan could be determined according to the results of genetic tests for inherited mutations for high risk groups. Lifetime risk of ovarian cancer in BRCA1 carriers are 40% to 50%, and 20% to 30% in BRCA2 carriers.[36] Therefore, bilateral salpingo-oophorectomy is recommended to reduce the risk of breast and ovarian cancer after childbearing has been completed, preferable before age 35 years.[49]

HNPCC, Lynch II syndrome, is an autosomal dominant disorder which predisposes to colorectal cancer, endometrial cancer, ovarian, gastric, small bowel, biliary/pancreatic, urothelial, skin, and central nervous system cancers. The lifetime risk of ovarian cancer is approximately 8-10%, with an average age of onset of 42 years.[50] Mutations in MSH2, MLH1, MSH6 and PMS2 account for most of families with Lynch syndrome.[51] Although the values of annual gynecologic follow up remain to be determined, it is usually recommended for HNPCC carrier women to have annual examination.[52,53]

The proportions of inherited mutations associated with ovarian cancer other than BRCA1 and BRCA2 are individually small, but together account for a significant proportion of cases.[43] Advances in genomic technologies accelerate the discovery of additional cancer susceptibility genes and increase the feasibility of comprehensive evaluation of multiple genes simultaneously at low cost. Using targeted capture and massively parallel sequencing, screening for germ-line mutations in 21 tumor suppressor genes was done from 360 women with primary ovarian, peritoneal, and fallopian tube carcinoma.[54] To date, at least 16 genes, including BRCA1, BRCA2, RAD51C, RAD51D, BRIP1, BARD1, CHEK2, MPE11A, NBN, PALB2, RAD50, MLH1, MSH2, MSH6, PMS2, TP53 have been associated with hereditary ovarian cancer.[43] Moreover, 30% of women with inherited mutations had no prior family history of breast or ovarian cancer, and 37% were diagnosed after age 60.[55] Therefore, some suggested that comprehensive genetic testing is warranted to all women with invasive ovarian carcinoma, regardless of age or family history.[43]

2. Epigenetic changes

Epigenetic mechanisms such as DNA methylation and histone modifications play important role in tumor initiation and progression as regulators of gene expression.[4] Since aberrant DNA methylation occurs early in cancer development and can be easily detected in clinical samples, measurement of methylation status provides great potential as a biomarker to detect early stage ovarian cancer.[56-58] Using sensitive methylation-specific PCR, methylation status of six tumor suppressor gene promoters, including BRCA1, RASSF1A, APC, p14ARF, p16INK4a, and DAPKinase were evaluated.[59] At least one or more hypermethylation was observed in tumor DNA obtained from 41 of 50 patients with ovarian or primary peritoneal tumors (82% sensitivity). In addition, hypermethylation was not found in nonneoplastic tissue or serum from 40 control women (100% specificity). However, there are few studies about global changes in DNA methlyation in ovarian cancer up to date. Genome-wide methylation profiles were generated by methylated DNA immunoprecipitation,

followed by promoter tilting array analysis for ovarian cancer cell line and ovarian surface epithelium samples. A panel of six genes (ARMCH1, ICAM4, LOC134466, PEG3, PYCARD and SGNE1) was obtained, and validation by direct measurement of DNA methylation showed the possibilitiy as a potent discriminator of cancer versus normal with a high AUC (0.98).[60]

MicroRNAs (miRNAs) are small (17-24 nt) non coding RNAs regulate many physiologic and pathological processes through control of gene expression.[61,62] It is now recognized that miRNAs are frequently dysregulated in malignancy, suggesting that they may act as a novel class of oncogenes or tumor-suppressor genes.[4] While most miRNAs are down-regulated in cancer, therefore suggested as tumor-suppressors, others are elevated and may act as oncogenes in ovarian cancer. Several miRNA profiling studies have demonstrated changes in miRNA patterns that take place during ovarian cancer development.[63-65] By using a microarray analysis, miRNA profiles were investigated from 69 ovarian malignant tumors, 15 normal ovarian samples, and 5 ovarian carcinoma cell lines.[63] miR-200a and miR-141 were elevated whereas miR-199a, miR-140, miR-145, and miR 125b1 were most significantly down regulated. Certain miRNAs could distinguish different subtypes of ovarian cancer. Approximately 50% of miRNAs reported in the previous study were found in other study from serous carcinoma samples.[64] From 23 patients from serous carcinoma and 8 from benign disease, the levels of several miRNAs such as miR-21, miR-125a, miR-125b, miR-100, miR-145, miR-16, and miR-99a were differentially expressed in more than 16 patients. These results show that miRNA deregulation is involved in ovarian carcinogenesis. In addition, miR-182 expression was significantly higher in STIC, possible precursor lesion for high-grade serous ovarian carcinoma.[66] Using microRNA profiling analysis, the researchers showed that miR-182 act an important role in early tumorigenesis of type II ovarian cancer.

miRNAs can also be detected in serum samples, and these intereting aspect makes miRNA as a useful detection biomarkers. Recently, it has been demonstrated that the miRNA signature of circulating tumor exomes is closely related with miRNA expression in primary tumor.[67] Circulating tumor exomes were isolated using magnetic beads and an antiEpCAM antibody. Levels of 8 miRNAs, including miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR214, were similar between cellular and exosomal miRNA, and these results suggest that miRNA analysis of circulating tumor exosomes could be used as diagnostic markers of ovarian cancer. Using a novel real-time PCR analysis from serum samples, miR-21, miR-92, miR-93, miR-126 and miR-29a were found up-regulated, while miR-155, miR-127, and miR-99b were observed down-regulated from ovarian cancer patient serum.[68] Furthermore, miR-21, miR-92, and miR-93, known oncogenes, were found to be significantly over-expressed in patients with preoperative normal CA-125 level. This suggests that miRNAs may be complementary to current detection approaches. Accumulating data showed that miRNAs are functionally involved in the pathogenesis of ovarian cancer and peripheral-blood derived miRNAs could be used as novel circulating biomarkers. However, there still exists no clear consensus on miRNA signatures associated to early dectection, prognosis or prediction to chemotherapy sensitivity in ovarian cancer.

3. Gene expression

DNA-microarray technology has enables us to analyze simultaneously the expression of thousands of genes in a small sample of tumor tissue. Thanks to powerful data analysis software, this high-throughput technology has made it possible to compare gene expression between normal and cancer and identify genes that are differentially regulated during cancer development. With clinical value for distinguishing normal ovarian tissue from ovarian tumors, gene expression profiling can provide useful information to discover novel biomarkers. The goal of such studies is to identify gene that are differentially upregulated in ovarian cancer, and to then determine whether these genes encode proteins that can be detected in the serum.[69]

Using oligonulceotide arrays, the researchers identified 275 genes predicted to encode proteins with increased/decreased expression in ovarian cancer.[70] Among them, serum levels of four proteins (osteopontin, kallikrein 10, secretory leukoprotease inhibitor, and matrix metallo-

proteinase-7) were significantly elevated in an independent set of 67 patients with ovarian cancer compared with 67 healthy controls. Combination these markers with CA-125 yielded sensitivity and specificity values ranging from 96% to 98.7% and 99.7 to 100%, respectively. Although further validations are required as a multi-analyte diagnostic test for ovarian cancer, combined clinical-genomic approach toward the identifying differentially expressed genes encoding putative secreted proteins provided the potentials for biomarkers. Analyzing gene expression in macrodissected formalin-fixed, paraffin-embedded samples from 5 high-grade stage I serous carcinomas and 5 stage I borderline tumors, FOL3, survivin, MCM3, E2Fs, and VTCN1 were overexpressed, and SYNE1, AKAP14, KNDC1, and DLEC1 were underexpressed in serous carcinoma.[71] These specific gene expression patterns in stage I serous carcinoma was associated with pathway such as cell cycle regulation, cell cycle-related cytoskeletal signaling, transcription-related chromatin modification, and kallikrein-related inflammatory signaling, which could be important in ovarian carcinogenesis and biomarker development.

In addition to role as a biomarker for early detection, gene-expression profiling can provide various information in ovarian cancer research, including prognosis, prediction of chemotherapy response, mechanisms of chemoresistance, characterization of different histologic and genetic subtypes. By using oligonucleotide microarrays, a 115-gene signature was identified from 68 patients with ovarian carcinoma.[72] This pattern was referred to as the Ovarian Cancer Prognostic Profile (OCPP), and validated in an independent set. The OCPP was more powerful prognostic factor for overall survival and disease-free survival than other known risk factors such as age, tumor stage, tumor grade, and debulking status. Moreover, chemo-response has been predicted by gene expression profiling.[73] Using a training set of 83 patients with advanced-stage serous ovarian carcinomas, the researchers documented a gene-expression model that predicted complete clinical response after platinum-based chemotherapy, and validated the results to an independent set of 36 patients. Gene expression profiles identified patients with ovarian cancer likely to be resistant to chemotherapy with greater than 80% accuracy. The investigators found expression signatures, SRC and Rb/E2F pathway, frequently found in chemo-resistant patients.

4. Whole genomic sequencing

DNA sequencing using dideoxynucleotide termination chemistry was first described by Fred Sanger in the 1970s and subsequently automated by capillary sequencing by Applied Biosystems in the 1990s. The first generation sequencing method was capable to sequence targeted regions of DNA spanning approximately 700 nucleotides at a time. By using this method, Human genome project was performed with sequencing all 3.2 billion bp at high coverage over a period of 10 years.[74] Recently, next or second generation sequencing have increased sequencing rates by orders of magnitude and driven down per base sequencing cost significantly. With the application of new technologies, it has became feasible to sequence the expressed genes,[75,76] known exons,[77,78] and complete genomes of cancer samples.[79-83]

Whole genomic sequencing provides the most comprehensive characterization of the cancer genome, leading to improvements in the diagnosis of cancer and the selection of cancer treatment. It is now widely expected that second generation sequencing will offer the in-depth characterization of the cancer cell genome and further advance the fields of pathogenesis of cancer and personalized oncology for patients.

The first whole cancer genome sequence was reported in 2008, comparing DNA from an acute myeloid leukemia with DNA from normal skin from the same patient.[79] Since then, rapid analysis of genetic alteration in a various tumor types has been done with advancing genomic technologies.[80-84] The whole genomic sequencing has the advantages than other methods in several aspects. First, discovery of chromosomal rearrangements could be feasible with whole-genomic sequencing. Previously, it was thought that chromosomal translocations were rare in epithelial tumors and observed mainly in hematologic malignancies. However, translocations such as transmembrane protease serine 2(TMPRSS2)-ERG translocations in prostate carcinoma and the echinoderm microtubule-associated protein like (EML4)-anaplastic lympho-

ma recerptor tyrosine kinase (ALK) translocations in non-small cell lung carcinoma were found in solid tumors.[85,86] Second, whole-genomic sequencing make it possible to detect other types of genomic alterations that have not been found using traditional method. Somatic mutations of non-coding regions such as promoters, enhancers, introns, and non-coding RNAs could be observed with whole-genomic sequencing.[87] Moreover, it provides non-biased approach to mutation detection than candidate gene sequencing.

Exome represents only approximately 1% of the genome, or about 30 Mb, vastly higher sequence coverage can be readily achieved using second-generations sequencing platforms with less time and cost than whole-genomic sequencing. In 2008, approach to identify the spectrum and extent of somatic mutations in pancreatic cancers was applied to samples from 24 patients with exomic sequencing.[88] Among 20,661 protein coding genes representing 99.6% of the known coding genome, 1,562 somatic mutations were detected. Most mutations were base substitutions, and a minority of small insertions and deletions, mutations at splice sites or the untranslated regions of these genes were also found. Each mutation was evaluated to identify the potential consequences of the mutations in pancreas cancer. For example, nonsense mutations that lead a stop codon prematurely end gene translation, and missense mutations that cause a change in the amino acid may or may not effect on protein function. Gene deletions or amplifications were less common than base substitutions. Based on analysis, 69 gene sets were genetically altered in the majority of the 24 cancer samples, and 31 of these sets could be categorized into 12 core signaling pathways and processes that were changed in 67 to 100% of the 24 cancer samples. In addition to pancreas cancer, Somatic mutations in non-small cell lung carcinoma were identified using whole-exome sequencing from 31 patients.[89] A novel gene CSMD3 was discovered as the second most common mutated gene in lung cancer. Second generation sequencing technologies revealed many genes not previously implicated as well as previously identified genes. Several highly mutated genes could be promising therapeutic targets in cancer therapy including ALK, CTNNA3, DCC, MLL3, PCDHIIX, PIK3C2B, PIK3CG and ROCK2.

Up to now, there are limited studies in fields of ovarian cancer with whole-genomic sequencing. With application whole-genomic sequencing to a patient with suspected cancer susceptibility, novel TP53 mutation was identified.[90] A patient presented with stage-2 breast cancer at age of 37 and stage IIIc ovarian serous cystadenocarcinoma at age of 39. However, she did not have a clear family history of cancer and no BRCA1 and BRCA2 mutations. At age 42, her ovarian cancer recurred and additional chemotherapy was done. While chemotherapy, acute myeloid leukemia was developed. Whole genomic sequencing of leukemia and skin DNA was performed on the Illumina platform using paired end reads with an average read length of 75 bp. A 3 Kb heterozygous deletion of TP53, encompassing exons 7-9, was detected in skin genome. Moreover, analysis of leukemia DNA showed a 17.6 Mb region of uniparental disomy on chromosome 17 that affected in homozygous deletion of exons 7-9 of TP53 in the leukemia genomes. The finding of the germline TP53 mutation has important clinical implications for patient's children. Whole genomic sequencing provided an unbiased survey of the genome and has ability to detect structural variants that could be missed by conventional methods. Furthermore, rare frameshift mutations in BRIP1 were identified and associated with increased risk of ovarian cancer.[91] The sequence variants identified through whole genomic sequencing of 457 general populations, were imputed to 41,675 Icelanders genotyped using SNP chips. The researchers found that a rare frameshift mutation, c.2040_2041insTT, in BRIP1 which behaves like a classical tumor suppressor gene.

The Cancer Genome Atlas Project has performed whole exome sequencing on ovarian cancer.[92] Analyzing DNA from 316 high-grade serous ovarian cancer samples and matched normal samples for each individual, 19,356 somatic mutations (about 61 per tumor) were annotated. High-grade serous ovarian cancer is characterized by TP53 mutations in almost all tissues (96%). BRCA1 and BRCA2 were mutated in 22% of tumors, due to a combination of germ-line and somatic mutations. Other significantly mutated genes including NF1, RB1, FAT3, CSMD3, GABRA6, and CDK12 occurred in 2-6% of cases. Mutational analysis

also showed that mutations in BRAF, PIK3CA, KRAS, and NRAS may be important drivers in high grade serous carcinoma. However, it has been demonstrated that these mutation spectrum in serous tumors was completely distinct from other ovarian cancer histological subtypes. For instance, clear cell types have few TP53 mutations but have recurrent ARID1A and PIK3CA mutations.[93-95] While CTNNB1, ARID1A and PIK3CA mutations were frequently found in endometrioid ovarian cancer histology, KRAS mutations were prevalent in mucinous types.[96]

Informations from whole genomic sequencing could be used for various purposes as well as biomarker for early detection. Whole genome sequencing gives insight into the heterogeneity of cancer. Cancer genomes are enormously diverse and complex. Variations between patients are considered as intertumor heterogeneity and categorized through different morphologic types, expression subtypes, and structural alterations by genomic sequencing.[97] Variation within a single tumor is referred to as intraumor heterogeneity, and has been found by heterogenous and composed of different clones that have different genomes.[97] Detailed sequencing studies of cancer have been failed to document recurrent mutations in cancer genes when mutational profiles are compared from patient to patient. It was reported that mutation of TP53 is more frequent in basal-like and HER2 subtypes, whereas PIK3CA mutation is observed to be overrepresented in luminal A tumors.[98-101] This emphasized the researchers should consider intratumor heterogeneity when designing experiments to detect novel mutation. Furthermore, many studies have showed that extensive genomic heterogeneity within tumors. Analyzing the metastatic progression of a basal-like breast cancer to the brain, approximately 50 coding mutations was found in the primary and metastatic tumors.[84] Few de novo mutations were observed in metastasis, but gross changes in allelic frequencies were observed, suggesting that minor subpopulations of cells with metastatic potential were pre-existing in the primary tumor. Intratumor variation at genomic level was found by showing allelic variation. Intratumor heterogeneity studies sequencing DNA from individual tumor cells require whole-genome amplification.[97] However, there still exists technical difficulty and limited reproducibility. Intratumor heterogeneity by single nucleus sequencing will provide clinical value in the early detection of tumor cells or tumor DNA in scarce clinical samples (urine, blood, fine-needle aspirates) and monitoring of circulating tumor cells after complete remission in the near future.

Therapeutic decisions could be determined by the results of genomic sequencing. The concept of personalized oncology began with the simultaneous regulatory approval of the anti-HER2-targeted monoclonal antibody therapeutic, trastuzumab for the treatment of HER2-overexpressing breast cancer.[102] Since then, several anticancer agents have been approved and more drugs have entered clinical trials based on biomarker profiles. For example, treatment with the inhibitors of the epidermal growth factor receptor knase (EGFR), gefitinib and erlotinib, lead to a significant survival benefit in patients with lung cancer whose tumors carry EGFR mutations.[103-105] Although published data are limited in describing clinical cancer samples with second generation sequencing, these new platforms accommodate large-scale gene sequencing than traditional Sanger sequencing, leading to determine unexpected sequence abnormalities as well as expected potential mutations. More candidate genes as prognostic and predictive biomarkers in ovarian cancer will be discovered.

The major challenge of whole genomic sequencing is computational, biological and clinical analyses of the genomic data. The computational analyses will assess reproducibility and statistical significance, the biological analyses will evaluate the association between pathways and functional relevance of mutated genes to cancer, and the clinical analyses will the effect of genome on incidence, histology, prognosis, and therapeutic response.[87] In addition, surgical resection specimens have been the mainstay of cancer genome analysis. In the near future, advances in sequencing technologies enables diagnosis from ever smaller samples, eventually including circulating tumor cells and free serum DNA.[106,107]

CANDIDATE GENE AS BIOMARKERS

1. TP53

TP53 gene encodes a transcription factor, and in response to a various cellular stresses, including DNA damages.

Activated TP53 protein binds to the regulatory sequences of target genes to initiate a cell cycle arrest.[108] TP53 mutations are most frequently observed genetic alterations in sporadic ovarian cancer. These mutations are found in 50-80% of high-grade serous carcinoma, but rarely seen in low-grade serous carcinoma, borderline tumors.[109] Considering high prevalence of TP53 mutations in tubal intraepithelial carcinoma, TP53 mutations occurred in early carcinogenesis.[33] TP53 mutations are suggested as poor prognostic factors and associated with early recurrence and poor response to platinum based chemotherapy and radiation.[110-112] Furthermore, the researchers found that TP53 could be a useful blood based biomarkers for detection of type II ovarian cancer.[113]

2. BRCA

BRCA1 and BRCA2 had somatic mutations in 3% of cases as well as germline mutations in whole exome sequencing results. About 20% of high-grade serous carcinoma samples had germline or somatic mutations in BRCA gene, and DNA hypermethylation caused inactivation of BRCA1 in a further 11% of cases.[92] Thus, genomic and epigenomic approaches could be predictive biomarker which is related to ovarian carcinogenesis. Furthermore, these defective homologous recombinations are known to highly responsive to poly-ADP ribose polymerase (PARP) inhibitor, providing a rationale for clinical trials of PARP inhibitors.

3. HER receptor family

While several HER-targeted therapeutics are US FDA approved for the treatment of various malignancies, there is no approval for therapeutic purposes to ovarian cancer up to now.[114] EGFR is over-expressed in 30-70% of high-grade serous carcinoma.[115] Activation of down-stream signaling pathway is known to mediate a various cellular responses such as cancer cell proliferation, survival, motility, and invasion. Increased EGFR expression has been correlated with poorer patient outcomes.[115] The association between HER2 overexpression and prognosis is still controversial.[116] Some suggested increased HER2/neu expression in ovarian cancer is associated with poor survival.[117,118] Overexpression of HER2/neu is observed in 20-30% of serous ovarian high-grade carcinoma, but rarely in low-grade and borderline tumors.[119]

4. AKT-2

Amplification of AKT-2 has been found in approximately 12% of type II ovarian carcinoma.[118] The significance of the PI3K/AKT pathway in ovarian cancer is well documented. This pathway is important in gene transcription, membrane trafficking, protein synthesis, and other processes, whereas abnormal activation of this pathway affects tumor initiation, progression, and invasion.[120,121] AKT2 amplification is suggested as independent prognostic factor for ovarian cancer, and PI3K/AKT/mTOR axis could be one of druggable targets.

STRATEGIES TO DISCOVER NOVEL GENE-BASED BIOMARKER IN OVARIAN CANCER

Considering dualistic model for ovarian tumorigenesis, the target of screening for ovarian cancer seems to be evident. Type I tumors are clinically indolent and not aggressive, progress by step-wise sequence. They represent only 25% of all ovarian cancer and are responsible for only 10% of ovarian cancer death.[122] In this context, efforts to discover novel biomarker for type I tumor are not urgently needed. Meanwhile, approximately 75% of all ovarian carcinoma and 90% of ovarian cancer death are from type II tumors.[122] Therefore, type II tumor should be targeted for screening. Until recently, a major limitation to studying ovarian cancer progression has been the lack of tissue for study. This is because almost type II ovarian cancers are not confined to ovary, even at their inception. Only 0.5% of type II tumors were confined to the ovary from the British Columbia Tumor Registry.[123] As fallopian tube carcinoma was included as part of disease spectrum associated with hereditary BRCA mutations, tubal intraepithelial carcinoma has been suggested as the possible precursor lesion for type II tumors.[124] We suggest the strategy to discover novel gene-based biomarkers in ovarian cancer, especially for high-grade serous carcinoma, the prototype of type II tumors (Fig. 1). First, much samples associated with precancerous lesion such as STIC are required through collaboration. And then, mutations in-

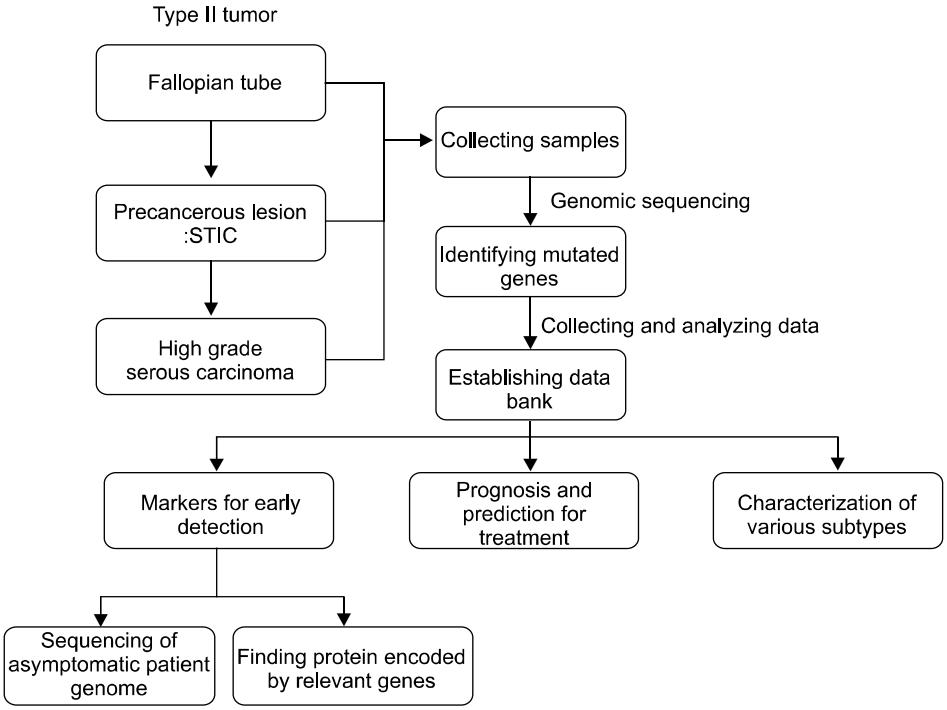

Fig. 1. Flow diagram for strategy to discover the novel gene-based biomarkers for high-grade serous ovarian carcinoma. STIC, serous tubal intraepithelial carcinoma.

volved in early carcinogenesis in type II tumors should be identified with genomic sequencing. Genomic sequencing provides the chance to discover genetic alterations occurred during early carcinogenesis before appearance of recognizable disease by comparing cancer and normal sample in same patient. After identifying and collecting the mutations found through genomic sequencing, the discovery of biomarkers for early detection could be performed in 2 directions. One is using genomic sequencing as a screening tool. Genomic sequencing will clarify the clonal evolution of ovarian cancer as well as provide time estimates of ovarian carcinogenesis. Comparing genomic sequencing for genes of asymptomatic patients with already identified mutations could be used as a novel method for early detection. The other way is finding the encoding protein that is relevant gene mutations in early carcinogenesis. Using proteomic technologies, proteins levels could be measured in asymptomatic patient serum.

CONCLUSIONS

Although the technologies of proteomics have been advanced rapidly, most of biomarkers have shown disappointing results and are not approved for asymptomatic populations. The reason is the complexity and dynamic range of serum and tissues biomarker levels. As a result, none of the proteins turned out to be better than CA-125 alone.[17] In particular, complexity and heterogeneity of ovarian carcinogenesis is considered as major challenges to discover novel biomarker. To overcome this problem, this review is toward the advances of novel biomarkers by genome sequencing of ovarian cancer because it affects translational, post-translational, regulatory and degradative processes of related RNA, proteins and metabolites.[125]

Although the pathogenesis of ovarian cancer is not well-recognized, a recent dualistic model for ovarian carcinogenesis shows two types of ovarian cancers based on clinical features and related gene mutations. Thus, novel biomarkers associated with gene mutations by genome sequencing has a potential to predict the risk of ovarian cancer, and to detect early-stage disease for improving its prognosis.[126,127] When we consider that the role of screening test for ovarian cancer is to detect precursor lesion and early-stage disease, novel biomarkers developed by new strategies such as genome sequencing will provide the best opportunity to reduce ovarian cancer mortality by increasing the detection rate of early-stage disease which can be cured by surgery with or without

adjuvant chemotherapy.

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