Biomarker Conservation in Primary and Metastatic Epithelial Ovarian Cancer

Krishnansu Sujata Tewari, M.D.,* Ainura S. Kyshtoobayeva, M.D., Ph.D.,† Rita S. Mehta, M.D.,*† Ing-Ru Yu, M.S.,† Robert A. Burger, M.D.,* Philip J. DiSaia, M.D.,* and John P. Fruehauf, M.D., Ph.D.*††

*Section of Hematology & Oncology, Division of Gynecologic Oncology, The Chao Family Comprehensive Cancer Center, University of California, Irvine–Medical Center, 101 The City Drive, Orange, California 92868; and †Oncotech, Inc., 1791 Kaiser Avenue, Irvine, California 92614

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INTRODUCTION

The management of women with ovarian cancer is complicated by the absence of satisfactory screening tools capable of reliably detecting early stage disease. Indeed, the poor survival associated with this malignancy is related to both the large tumor burden often present at the time of diagnosis, and the presence of chemoresistant clones that stubbornly persist after surgical cytoreduction and postoperative adjuvant systemic therapy. It has been postulated that tumor heterogeneity, acquired during tumor progression from the nascent malignant clone to clinically detectable disease, contributes to the poor prognosis of ovarian cancer patients [1].

Prognostic factors associated with ovarian cancer may be pathologic, clinical, or biologic [1, 2]. Pathologic factors that may impact on survival include nuclear grade, cell type (e.g., clear cell carcinoma), cellular architecture (e.g., papillary patterns), and the presence of occult residual disease following initial debulking surgery. On the other hand, clinical prognostic factors include surgical FIGO stage at diagnosis, volume of ascites, elevated pretreatment serum levels of cancer antigen 125, patient age, performance status, and the presence of measurable disease during systemic treatment and surveillance periods.

Biologic factors, or biomarkers, that have been correlated with prognosis in ovarian cancer may be grouped into five categories. Category one consists of cell growth regulators, such as the tumor suppressor gene product, p53. Category two, proliferation factors, includes the oncogene product HER2 and the epidermal growth factor receptor (EGF-R). Category three, gene products associated with drug resistance, includes the multidrug resistance-1 gene product, MDR1. Category four, angiogenic factors, includes the endothelial cell antigen (CD31), the key promoter of angiogenesis, vascular endothelial growth factor (VEGF), and the potent inhibitor, thrombospondin-1 (TSP-1). Category five, nuclear DNA content, includes the S-phase fraction and DNA index (i.e., ploidy). Acquisition of an adverse biomarker profile is thought to occur as a result of random mutational events in somatic cells.
The Goldie–Coldman hypothesis has been advanced to explain the relationship between mutational events and the development of tumor resistance to chemotherapy [3]. This hypothesis predicts that random mutations occur continuously that confer drug resistance to selected populations of cells [4, 5]. What is not clear is when these events occur during the course of disease progression. Because the nascent events related to the development of epithelial ovarian carcinoma are poorly understood, it is unclear whether biomarker changes occur early in disease progression, whether they occur after metastases have been established, or whether these mutational events occur continuously. The clinical importance of biomarker divergence is related not only to tumor heterogeneity that may confer resistance to chemotherapy, but also to the emergence of aggressive subsets of tumor cells that influence the rate of disease progression in a given patient.

We therefore chose to evaluate a series of biomarkers to determine whether, in cases of advanced disease, the primary tumor was biologically representative of the metastatic lesions, or whether clonal divergence of these specific markers had occurred between sites. We evaluated biomarkers from each of the five categories described above. The analysis was initially performed on a large unselected population of primary and metastatic specimens and then an assessment of paired synchronous and metachronous cases was undertaken.

**MATERIALS AND METHODS**

Tissues harvested from women with advanced stage or recurrent epithelial ovarian cancer were evaluated by immunohistochemistry for expression of p53, HER2, EGF-R, MDR1, and CD31 and by flow cytometry for DNA content.

**Immunohistochemistry**

*p53.* Wild-type p53 represents a tumor suppressor gene product. p53 is the most commonly mutated gene in cancer and normally suppresses cell cycle progression at the G1 cell cycle checkpoint when cells have been damaged by radiation or antineoplastic agents. It has an inhibitory effect on proliferation in normal tissues. In neoplasia, the accumulation of mutant p53 results in the inactivation of the mechanisms responsible for the suppression of proliferation. Point mutations in p53 lead to its cellular accumulation due to decreased rates of ubiquitination and an increased protein half-life. This accumulation makes it possible to detect most mutant forms of the protein by immunohistochemistry.

Mouse monoclonal IgG2a clone DO-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to stain specimens utilizing standard protocols on a Ventana automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ). Only nuclear staining was reported as a positive result.

**HER2.** HER2 is a proto-oncogene product encoded on chromosome 17. HER2 is a 185-kDa transmembrane protein that has sequence homology with the epidermal growth factor receptor. HER2 is involved in positive cellular growth control. Amplification or overexpression of HER2 is found in breast, ovarian, and other epithelial adenocarcinomas.

Mouse monoclonal IgG1 clones CB11 or E2 4001 (Neomarkers, Inc., Union City, CA) were used to stain tissues utilizing standard protocols on a Ventana automated immunohistochemistry system. Membrane staining only constituted a positive result.

**EGF-R.** Epidermal growth factor receptor is a 175-kDa transmembrane glycoprotein that has an extracellular EGF binding domain and a cytoplasmic domain with tyrosine kinase activity. Overexpression of this antigen can occur in breast, prostate, ovarian, brain, lung, and squamous cell carcinomas.

Mouse monoclonal IgG1 antibody clone Ab-10 (Neomarkers, Inc.) was used to stain tissues utilizing standard protocols on a Ventana automated immunohistochemistry system. Membrane and/or cytoplasmic staining were reported as positive.

**MDR1.** MDR1 is a 170-kDa glycoprotein (P-170) that functions as a transmembrane efflux pump. MDR1 is essential to the most widely characterized mechanisms of drug resistance. It is expressed by a large number of normal tissues, including the proximal renal tubules, hepatic ducts, and bowel lumen. The primary function of the protein is to remove potentially harmful substances from cells. In neoplastic tissues, the expression of MDR1 has been correlated with resistance to chemotherapeutic agents, such as paclitaxel, doxorubicin, etoposide, and mitomycin C.

Mouse monoclonal IgG1 clone JSB1 (BioGenex Laboratories, San Ramon, CA) was used to stain specimens utilizing standard protocols on a BioGenex automated immunohistochemistry system. Membrane, cytoplasmic, and/or perinuclear staining patterns constituted positive results.

**CD31.** CD31 is an endothelial cell surface antigen that is associated with blood vessel structures. Angiogenesis is required for tumor growth at the primary site and for successful implantation at metastatic sites. Increased CD31 staining reflects neovascularization and angiogenesis.

Microvessel density based on quantification of CD31 immunostaining using mouse anti-human CD31 (Becton–Dickinson Co., San Jose, CA) was performed on a Ventana automated immunohistochemistry system; a value of 40 vessel counts per 200× field was selected for distinguishing between normal and overexpression of the marker.

**Flow Cytometry**

**S-phase fraction (SPF).** SPF represents the proportion of tumor cells that are in the DNA synthetic phase of the cell cycle and thus is a measure of cellular proliferation.

**DNA index.** The DNA index was reported as either DI = 1.0 (diploid) or DI > 1.0 (aneuploid). Fresh malignant tissues containing a minimum of 20,000 tumor cells (approximately 100 mg or 4 mm³) were processed on a FACScan flow cytom-
eter (Becton–Dickinson, Mountain View, CA). Microspheres were used to establish the limits of variability in confidence values. Fresh mononuclear cells isolated from a single donor were used as the normal control and to verify the location of nuclei with normal DNA content. The abnormal control consisted of a hyperploid human breast carcinoma cell line, MCF7-40F. A DNA fluorescence histogram was generated from which the S-phase fraction.

**Statistical methods.** Statistical differences between groups were determined using the \( \chi^2 \) test or the Fishers exact two-sided test run on the In-Stat (San Diego, CA) PC-based software program.

**RESULTS**

**Clinical Material**

From 1993 to 1998, a series of 3173 ovarian carcinoma specimens submitted to Oncotech, Inc., in Irvine, California, from both regional and out-of-state institutions was evaluated for prognostic marker expression and *in vitro* drug resistance testing. All tissue samples were obtained from women with advanced primary or recurrent FIGO Stage III/IV disease. Metastatic sites from which malignant tissue were retrieved included uterine serosa, omental deposits, intestinal serosa and mesentery, bladder serosa, liver capsule, subdiaphragmatic surfaces, pelvic and periaortic lymph node chains, splenic hilum, and the peritoneal surfaces of the pelvic sidewall and cul-de-sac.

All metachronous lesions were temporally separated by 5 years or less. Due to the large number of referral centers, other clinical data such as age, performance status, systemic treatment regimens, response rates, and survival were not uniformly obtainable.

**Unselected Population of Primary and Metastatic Cases**

Of the 3173 specimens received, 1036 represented primary ovarian carcinomas and 2137 were obtained from metastatic sites. All malignant tissues were immunostained for MDR1. Only 12% of the primary and 10% of the metastatic specimens exhibited measurable levels of MDR1 expression. Similarly, approximately 70% of the specimens in each group were immunostained for HER2, with overexpression detected in 12% of primary specimens and in 11% of metastatic lesions. None of these comparisons were significantly different (Table 1).

Approximately 70% of the specimens in each group also underwent analysis for EGF-R by immunohistochemistry. As shown in Table 1, of the primary tumors \( (n = 723) \), 26% were found to be positive for EGF-R, compared to 33% of the metastatic lesions \( (n = 1414) \) \( (P = 0.0013) \). Approximately 45–50% of the specimens were immunostained for p53, with positive staining identified in 55% of the primary tumors \( (n = 496) \) and in 60% of the metastatic tissues \( (n = 964) \). This comparisons was not significantly different.

Eighty percent of the primary tumors and 85% of the metastatic tissues were examined by flow cytometry. No significant differences in the level of aneuploidy (approximately 73%) or in SPF >7% (57–58%) were detected between the two groups. These data are also summarized in Table 1.

Preliminary results utilizing CD31 immunostaining as a biomarker for microvessel density in 25 primary tumors and 17 metastatic lesions appear in Table 2. Angiogenesis was increased in metastatic sites compared to the primary tumor \( (P = 0.0012) \).

**Paired Synchronous Primary and Metastatic Cases**

The expression of p53 \( (n = 23) \), HER2 \( (n = 38) \), and MDR1 \( (n = 46) \) was not significantly different between

### TABLE 1

| Marker      | Primary   | Metastatic |
|-------------|-----------|------------|
| N           | % Positive | % Negative | N           | % Positive | % Negative | P value |
| MDR1        | 1036      | 12         | 88         | 964        | 10         | 90       | N.S.    |
| p53         | 496       | 55         | 45         | 1444       | 11         | 89       | N.S.    |
| HER2        | 730       | 12         | 88         | 1414       | 33         | 67       | 0.0013  |
| EGF-R       | 723       | 26         | 74         | 1821       | 73         | 27       | diploid |
| DNA ploidy  | 832       | 73         | aneuploid  | 27         | diploid    |          |         |
| S-phase >7% | 832       | 57         |            | 43         |            |          |         |

### TABLE 2

| Vessel counts per 200x field (CD31) |
|-------------------------------------|
| Primary \( (n = 25) \)              |
| Vessel counts per 200x field (CD31) |
| <40                                 |
| ≥40                                 |
| 38%                                 |
| 5%                                  |
| 21%                                 |
| 36%                                 |

*Note. Fishers exact two-sided test: \( P = 0.0012 \). Percentages listed are percentages for the entire population of 42 specimens.*
primary and metastatic sites from the same patient. Approximately 64% of primary tissues and 59% of specimens from metastatic sites had immunodetectable levels of p53. Similarly, approximately 12–13% of primary tissues and 10–11% of metastatic specimens were found to express MDR1 or HER2, as depicted in Fig. 1.

DNA content expressed as ploidy status was not appreciably different between primary and metastatic sites from the same patient (n = 31 paired lesions). As shown in Fig. 2, approximately 87% (n = 27) of the primary cases were aneuploid, while 77% (n = 24) of the metastases exhibited aneuploidy.

**PAIR METACHRONOUS CASES**

The expression of p53 (n = 51), HER2 (n = 70), and MDR1 (n = 154) differed minimally between tissues obtained from the same patient at the primary surgery and at the time of recurrence. Nearly 71% of the primary cancers had detectable levels of p53, which was maintained in approximately 68% of the recurrent tumors. In addition, MDR1 and HER2 were expressed in less than 10% of paired primary and recurrent lesions, as shown in Fig. 3.

DNA content, expressed as ploidy status, also did not diverge significantly between these matched primary and recurrent tumors (n = 66). As depicted in Fig. 2, 74% (n = 49) of the primary tumors were aneuploid, of which 79.5% (n = 39) retained their aneuploid status at recurrence; 15% (n = 10) of the primary aneuploid tumors were found to be diploid at relapse.

**DISCUSSION**

In this study we found that the biomarkers p53, HER2, MDR1, and DNA ploidy were conserved between primary and metastatic sites for a large unpaired population and for smaller paired synchronous and metachronous groups. These data indicate that for the biomarkers selected and the cases examined, little clonal divergence occurred between primary and metastatic sites. The concepts of somatic mutation and clonal divergence during malignant transformation and tumor progression provide the basic platform for modeling cancer development and for designing therapeutic regimens of combination chemotherapy.

The *somatic mutation hypothesis*, formulated by Boveri in 1914, was an early attempt to explain the origin of cancer [6]. He suggested that a single abnormal “chromosome combina-
tion” would be sufficient to confer a malignant phenotype to a cell. This theory has been supported by the discovery of the Philadelphia chromosome aberration (Ph) in patients with chronic myelogenous leukemia [7], the production of a distinct immunoglobulin from a specific multiple myeloma [8], and the random inactivation of the second X chromosome during embryogenesis of female mammalian somatic cells (i.e., Lyonization) which confers mosaicism for heterozygous X-linked genes [9].

The clonal origin of epithelial ovarian carcinoma was verified by Jacobs et al. at Duke University in 1992 [10] and by Abeln et al. at the University of Leiden in The Netherlands in 1995 [11] through separate analyses of loss of heterozygosity, p53 mutation, and X-chromosome inactivation and by DNA flow cytometric studies. While clonal mutations of somatic cells explain the origins of cancer, with the advent of molecular analysis it has become clear that ongoing genetic changes are involved in tumor progression, resulting in tumor heterogeneity.

Both human and animal tumors exhibit extensive heterogeneity in cellular morphology, cell surface markers, and nuclear chromosomal content. Biologically and clinically, such heterogeneity translates into differences in tumor growth rate, metabolic characteristics, immunogenicity, and sensitivity to and recovery from exposure to irradiation and cytotoxic drugs [8, 12]. If tumor cell populations derive from a cenancestor or single transformed cell, then biological divergence during cell division and proliferation must take place at some point along the spectrum that extends from the clonal origin of neoplasia to the clinical declaration of malignancy.

Nowell’s model to explain the genetic instability of neoplastic cells has been outlined succinctly by Ruddon [8, 13]. In this model, malignant cells experience several “evolutionary” changes that produce genetically variant cells, represented by aneuploidy in many tumors. Due to metabolic disadvantages and/or immunologic rejection by the host, many of these aberrant cells are eliminated. However, those with a selective advantage will proliferate and become predominant. With time, there is a sequential selection of subpopulations of cells with increasingly abnormal karyotypes, states of differentiation, and metastatic potential.

The phenomenon of clonal divergence may be explained by the Goldie–Coldman hypothesis, a mathematical model based on the concept that biological and clinical characteristics of tumors may be the sequelae of spontaneous mutations [3–5]. If this model is correct, early detection should lead to improved treatment outcomes on the basis that fewer mutations causing drug resistance and aggressive behavior would be present in smaller lesions. It is therefore relevant to determine when clonal divergence occurs. Does it operate constitutively at all sites, or is there a genetic window of susceptibility that can be defined, characterized, and manipulated or perturbed? Are there consistent phenotypic characteristics at a specific stage of disease that may confer vulnerability to a single chemotherapeutic or immunotherapeutic approach? In terms of gynecologic malignancies, Dembo has considered these questions carefully in the setting of trophoblastic neoplasia, a disease for which drug resistance still constitutes a clinical hurdle, despite its near complete curability in most cases [14]. The question is of paramount importance with respect to epithelial ovarian cancer which for the most part carries a grim and devastating prognosis. Recent advances in laboratory medicine have made it possible to begin to identify biomarkers that may characterize the prognosis and drug response profiles of individual patients.

In experimental designs similar to our own, previous investigators have attempted to describe tumor heterogeneity as a function of the prognostic significance imparted by various biomarkers in primary tumors and metastases. Reporting in 1993 from the Medical College of Pennsylvania, Zangwill and co-workers noted a 48% incidence of ovarian tumor heterogeneity when primary and metastatic samples from 19 patients were examined for DNA content [15]. In contrast, in 1994 Kaern and colleagues from the Norwegian Radium Hospital in Oslo communicated their results of an analysis of 119 tissue samples retrieved from 42 patients with metastatic ovarian carcinoma [16]. Fresh tumor material was studied by flow cytometry and 81% of the metastases were found to have a stable DNA configuration when compared with the primary tumors. Similarly, in 1997 Brinkhuis and co-workers from the Free University Hospital in Amsterdam examined primary ovarian tumors and their omental metastatic deposits and found no significant differences between the sites for mean nuclear area, mean nuclear volume, or mitotic activity index [17].

Although Calugi et al. described clonal divergence in a patient for whom a p53 point mutation was present in both omental and lymph node metastases but not in the primary ovarian tumor [18], subsequent larger studies, including our own, have failed to support the hypothesis that primary and metastatic sites exhibit differential p53 profiles. Specifically, in 1996, Daidone and co-workers from the Istituto Nazionale in Milan examined cancer tissue from primary and synchronous metastatic sites from 61 untreated women with ovarian carcinoma and were unable to detect a difference in p53 immunostaining; in addition, the aneuploidy frequency and DNA indices were conserved in primary and metastatic lesions for a given patient [19]. These investigators did, however, observe markedly heterogeneous proliferative profiles (based on tritiated thymidine labeling) in primary and synchronous metastases, predominantly in omental lesions.

Kimball and colleagues from the Women’s Cancer Center of Northern California performed a flow cytometric analysis of malignant tissues from 35 women with metastatic ovarian carcinoma in 1997 [20]. Interestingly, although the DNA ploidy distribution frequency of peritoneal metastases mirrored that found in the primary tumor, both were significantly different from the DNA ploidy distribution frequency found in metastatic lymph nodes. Although we were unable to detect a
difference in DNA ploidy distribution between primary and synchronous metastases, we did not subcategorize the metastatic lesions by site in our analysis.

In addition to studying synchronous primary and metastatic tissues, our study design included paired metachronous ovarian malignancies. We have identified three published works where a similar scientific endeavor was undertaken. In dramatic contradiction to our results, in 1995, Venesmaa and colleagues from the University of Helsinki in Finland examined malignant tissues of 26 patients (9 diploid, 6 aneuploid, 11 heterogeneous) retrieved before and after treatment with cytotoxic chemotherapy and observed that the DNA ploidy status changed in 58% of the cases [21]. This group noted that the patients who enjoyed a statistically significant improvement in 5-year survival were those in whom a diploid tumor was maintained or achieved. In 1996, Zanetta and investigators from the Mayo Clinic studied 16 advanced ovarian cancer cases for which malignant tissue from the primary and second-look surgeries was available: the concordance in DNA ploidy reached 63% [22]. Goff and co-workers at the University of Washington immunophenotyped the malignant tissues retrieved at primary surgery and at reassessment laparotomy of 23 women with advanced ovarian cancer in 1998 [23]. A comparison of multiple biomarkers (including p53, EGF-R, c-erbB-2, and Ki67-defined cellular proliferation antigen) revealed that the only significant change was in the Ki67-defined cell proliferation rate, which was markedly reduced in tumor obtained at the time of second-look laparotomy.

Of course, our inability to identify biologic variations of p53, HER2, MDR1, and DNA ploidy between primary and metastatic lesions in both our large unselected analysis, and in the paired synchronous primary–metastatic cases may simply reflect that we did not study the correct markers linked to clonal divergence. However, if clonal divergence were an ongoing phenomenon constituting random genetic events, then it is unlikely that separate lesions would predominantly/always change in the “same direction” (i.e., experience equivalent/homologous mutations) or that certain genes would be uniformly resistant to mutation (e.g., p53, HER2, MDR1) while others would be uniformly susceptible (e.g., occult biomarkers).

Perhaps clonal divergence occurs preferentially before the metastatic event takes place. It is likely that early in the development of a solid tumor malignancy, random mutational events occur with greater frequency during a time when processes such as basement membrane invasion, anchorage independence, and angiogenesis have been initiated, i.e., when the tumor is relatively biologically immature and is “testing” the host environment and its own ability to escape or reckon with immunosurveillance mechanisms. This may be analogous to Stage I disease when the cancer is confined to the ovary and perhaps relatively genetically unstable compared to when it has become aggressive and declared its malignant potential clinically (i.e., metastasized). Thus, whatever random mutational events have taken place to confer biologic heterogeneity probably have occurred early in the disease, prior to metastasis or recurrence. If this is indeed the case, then the study of tissues from early stage cases may provide greater insight. Indeed, Schueler et al. identified the DNA index as a primary prognostic factor for disease-free survival in early stage (I–IIA) epithelial ovarian cancer [24]. Ideally, it would be important to compare the biomarker profiles of early stage cases with those of advanced or recurrent tumors.

There is accumulating evidence that angiogenesis plays a central role in ovarian cancer disease progression. Although clearly limited by small numbers, our preliminary results examining microvessel density as a function of CD31 expression would support the contention that the molecular differences between primary and metastatic lesions may represent not only clonal divergence of EGF-R and other (as yet unknown) biomarkers, but also a phenotypic adaptation of the tumor to a new (i.e., extraovarian) site. It is presumed that ovarian epithelial tumor angiogenesis follows malignant transformation of predisposed ovarian surface epithelium. Current models suggest that microvessel invasion into the ovarian stroma is enhanced by endothelial cell release of proteolytic enzymes and disruption of intercellular adhesion, potentially triggering the release of tumor cells into lymphatic and vascular spaces. These metastatic cells are ideally suited to effective implantation at distant sites by virtue of their proangiogenic phenotype. As in other tumors studied, VEGF expression has been detected in human ovarian cancer [25] and a direct relationship has been found between VEGF expression and microvessel density [26]. Investigators have hypothesized that the “angiogenic” activity within an ovarian cancer should directly influence its metastatic potential and biologic aggressiveness. However, the impact of angiogenic activity on clinical outcome for patients with ovarian cancer has yet to be convincingly determined. Gasparini and co-workers recently reported on 60 women with advanced ovarian carcinoma treated with standard surgery and chemotherapy, in which CD31 expression was a negative prognostic factor for survival [27]. The same association was demonstrated by Hollingsworth and colleagues in their study of CD31 expression in 43 similar patients [28]. These and our preliminary data have encouraged us to pursue our angiogenic analyses with CD31 and, potentially, VEGF and TSP-1.

Malignant transformation is based on complex multifactorial nonlethal mutations that are likely to be conserved in progeny cells because they confer a survival advantage. Some of these mutations, such as those affecting p53, may predispose the cell to further mutations due to loss of control over DNA repair and cell cycle check points. This is consistent with the proposal by Goff et al., who stated that “...lack of a recognizable pattern of [marker] expression emphasizes the underlying biologic complexity of ovarian cancer” [29]. One of the important considerations we sought to address in our investigation is whether site-to-site biological variation of the specific biomar-
kers we tested may contribute to the difficulty in interpreting prognostic information. From our data we may conclude that, when metastatic sites are considered together, this would not appear to be the case. Perhaps, as Kimball et al. have suggested, lymphatic metastases are biologically different than those resulting from intraperitoneal spread or hematogenous dissemination [20].

Although we believe that our pathologic material is representative of an unselected or general population of epithelial ovarian cancer cases, we acknowledge that the absence of clinical data limits the present study. In addition, the inability to evaluate the paired cases for EGFR expression is unfortunate as this was the only biologic marker for which statistically significant heterogeneity was demonstrable in the larger population analysis we conducted. Nevertheless, the results and interpretations are thought provoking and should prompt further study on the subject of clonal divergence in ovarian cancer, especially in early stage cases. Furthermore, we recognize the importance of studying other biologic markers such as Ki67-defined antigen, and other as yet uncharacterized relevant occult genomic sequences, which we anticipate will be forthcoming as the collaborative efforts of the National Institutes of Health and the Department of Energy continue in mapping the entire human genome.

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