Specific Genes Expressed in Association with Progesterone Receptors in Meningioma

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Abstract

An association between hormones and meningioma has been postulated. No data exist that examine gene expression in meningioma by hormone receptor status. The data are surgical specimens from 31 meningioma patients undergoing neurosurgical resection at Brigham and Women's Hospital from March 15, 2004 to May 10, 2005. Progesterone and estrogen hormone receptors (PR and ER, respectively) were measured via immunohistochemistry and compared with gene expression profiling results. The sample is 77% female with a mean age of 55.7 years. Eighty percent were grade 1 and the mean MIB was 6.2, whereas 33% and 84% were ER+ and PR+, respectively. Gene expression seemed more strongly associated with PR status than with ER status. Genes on the long arm of chromosome 22 and near the neurofibromatosis type 2 (NF2) gene (22q12) were most frequently noted to have expression variation, with significant up-regulation in PR+ versus PR- lesions, suggesting a higher rate of 22q loss in PR- lesions. Pathway analyses indicated that genes in collagen and extracellular matrix pathways were most likely to be differentially expressed by PR status. These data, although preliminary, are the first to examine gene expression for meningioma cases by hormone receptor status and indicate a stronger association with PR than with ER status. PR status is related to the expression of genes near the NF2 gene, mutations in which have been identified as the initial event in many meningiomas. These findings suggest that PR status may be a clinical marker for genetic subgroups of meningioma and warrant further examination in a larger data set. [Cancer Res 2008;68(1):314-22]

Introduction

Meningiomas account for ~20% of all intracranial tumors in males and 38% in females, yet little is known about the risk factors associated with these lesions (1).⁵ The prevalence of meningioma is estimated to be ~97.5/100,000 in the United States with >150,000 individuals currently diagnosed with this tumor. Data from the Central Brain Tumor Registry of the United States reveal an age-adjusted incidence rate (per 100,000 person years) of 5.04 and 2.46 for females and males, respectively.⁵

An association between hormones and meningioma risk is suggested by a number of findings including the increased incidence of the disease in women versus men (2:1),⁵ the presence

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of estrogen receptors (ER) and progesterone receptors (PR) in some meningiomas (1–8), a potential association between breast cancer and meningiomas (9), an association in some studies between exogenous or endogenous hormones and meningioma risk (1, 10–16), as well as reports that meningiomas may change in size with menstrual cycle phase, pregnancy, and menopausal status (17–20).

Given these observations, a large number of investigators have examined both the prevalence and function of ER and PR in meningioma, with these tumors in general showing a low expression level of ER and a high level of PR (1-8). Although the specific role of both types of receptor as well as their isoforms remains unclear, there is growing evidence to support a role for PR. Several studies have reported that PR+ tumors are more likely to be benign and to have a lower rate of recurrence (5, 7, 8) whereas evidence for the functional role of PR has been obtained from laboratory analyses including in vivo evidence in nude mice implanted with human meningioma and treated with the antiprogestin RU-486 (21), although results from clinical studies remain uncertain (21-23). The prevalence and function of ER in meningiomas remain a more controversial topic and efforts to use antiestrogen drugs such as tamoxifen as a treatment option for meningioma have led to inconclusive results at present (24). To further examine the role that ER and PR might play in meningioma tumors, we carried out microarray-based expression profiling of 31 meningioma specimens from a hospital-based series using oligonucleotide arrays representing >38,000 different genes. Few gene expression analyses exist using meningioma specimens (25-27). Current studies examine expression by grade (25), anatomic location (27), and preparation (26). To our knowledge, none have examined expression by either ER or PR status. Given the strength of the epidemiologic and clinical evidence indicating that hormone exposure is an important risk factor for meningioma development as well as the fact that these receptors may be an important clinical target for treatment of this lesion, we examine gene expression by receptor status in a hospital-based series of meningioma.

Materials and Methods

The data are surgical specimens collected from 31 patients without a history of neurofibromatosis type 2 (NF2) undergoing first neurosurgical resection of an intracranial meningioma at Brigham and Women's Hospital from March 15, 2004 to May 10, 2005. Written consent was obtained from study subjects and approval for this study was granted by the Institutional Review Board of Brigham and Women's Hospital. Cases were included if (a) patient consent was obtained, (b) surgery was done on a weekday with

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⁵ Central Brain Tumor Registry of the United States (CBTRUS); http://www.cbtrus. org/reports//2005_2006/2006report.pdf, page 21, Fig. 10. Accessed January 8, 2007.

laboratory staff available to receive the specimen, and (c) sufficient amounts of RNA were available from the tumor samples remaining after review by the surgical pathologist. A total of 62 eligible (i.e., nonrecurrent) cases were available of whom 31 (50%) met the three criteria listed. A uniform histologic review was then done by the study pathologist (J.C.) using the WHO classification scheme for meningioma (28).

Gene expression analyses. If permitted by surgical pathology, tumor tissue was snap frozen at the time of surgery and stored at -80° C in the

Brain Tumor Tissue Bank at Brigham and Women's Hospital. Extraction of RNA from these fresh-frozen tumor specimens was done. The mRNA was reverse transcribed to generate cDNA, which was then biotinylated and hybridized to Affymetrix U133 plus 2.0 expression arrays (Affymetrix, Inc.). This array contains all the probe sets from the U133 Set as well as an additional 6,500 to measure a total of 47,000 transcripts. Each probe set contains eleven 25-mer probes selected from the same target region of a gene, and each probe in turn contains a perfect match probe and a



Figure 1. Heatmap by study subject PR status (horizontal axis) and probe (vertical axis).

mismatch probe. The expression values were computed from the MAS 5.0 algorithm (29), which uses a Tukey biweight formula to give lower weights to outliers in averaging the values across the probes in a probe set. Arrays were normalized for array-to-array variation by setting the trimmed mean (excluding the extreme 2% of the data in each tail) to be 100 for all arrays. Those probe sets given the "absent" call by the algorithm in more

than three fourths of the samples were filtered out because they are likely to be nonexpressed in these samples; 28,925 transcripts remained after this filtering. Hierarchical clustering was done using the distance measure 1 - r (where *r* is the Pearson correlation coefficient) and average linkage.

To identify differentially expressed genes between phenotypes (ER or PR status), the groups were compared using the t test. To assess significance



Figure 2. Heatmap by study subject ER status (horizontal axis) and probe (vertical axis).



Figure 3. Dendrogram using 29,000 genes.

of these genes, we computed the false discovery rate (FDR; ref. 30) as described by Storey et al. (31). This is the percentage of genes called significant that turn out to be false leads. For example, a FDR of 10% for a gene means that among all genes that are as significant or more significant, only 10% are likely to have been nondifferentially expressed but were incorrectly found to be differentially expressed. When multiple hypotheses are being tested simultaneously, this measure of the rate that the significant features are truly null is generally more helpful than the false-positive rate for the truly null features (P value). Unless otherwise stated, in these analyses a FDR of 5% was used to define statistical significance with respect to differences in gene expression by receptor status. Pathway analyses were done using software developed by our group in an effort to define differentially expressed sets of genes that belong to known genetic groups or pathways (i.e., DNA repair pathway, etc.). The statistical language R⁶ and packages from Bioconductor Project⁷ were used for all analyses of the data with the exception that Statistical Analysis System (SAS) software version 9.1 was used to carry out descriptive analyses (proportions, means, and SDs) as well as bivariate analyses (Fisher's exact test; ref. 32).

Immunohistochemistry analyses. Immunohistochemical staining was done on 5- μ m sections of the formalin-fixed paraffin-embedded material using the Envision+ DAB system (DAKO) per manufacturer's protocol along with mouse monoclonal antibodies against ER (1:100 dilution; DAKO), PR (1:200 dilution; DAKO), and Ki-67 (1:200 dilution; DAKO). After routine deparaffinization/rehydration of sections and before quenching of endogenous peroxidase activity, antigen retrieval was done in a pressure cooker by heating in 10 mmol/L Na-citrate (pH 6.0) to a temperature of 125°C for 30 s followed by gradual cooling to 90°C over 30 min. Primary antibodies or IgG isotype–negative controls were diluted in TBS with 1% bovine serum albumin and were applied for 40 min at room temperature. All washes were done with TBS with 0.1% Tween 20. Following the Envision+ DAB detection

Immunohistochemical stains for ER, PR, and Ki-67 (MIB) were evaluated by a pathologist blinded to the results of the gene expression profiling. Staining was visually assessed in at least 1,000 tumor cells in two separate fields using a $40 \times$ objective in the areas of greatest immunopositivity. For MIB, staining was resported as a proliferation index (percent of cells proliferating); this was calculated by dividing the number of tumor cells showing positive nuclear staining by the total number of tumor nuclei. For ER and PR, the immunostaining was assessed on a semiquantitative scale of 0 to 4 as follows: score 0, negative; score 1, positive <10%; score 2, positive 10% to 49%; score 3, positive 50% to 90%; score 4, positive >90%.

Results

The sample is 77% female with a mean age of 55.7 years. Eighty percent of the meningioma samples were defined as grade 1 and 20% as grade 2 (no lesions were categorized as grade 3 or malignant); the mean MIB was 6.2 whereas 33% and 84% of samples were ER and PR positive, respectively. Twelve of the lesions are classified as fibrous, seven as transitional, nine as meningothelial, and one each as secretory and psammomatous. ER and PR status was not associated with sex, age at diagnosis, MIB, grade, or histologic subtype. Heatmaps for the top 100 genes are presented by PR and ER status, respectively, in Figs. 1 and 2, whereas Fig. 3 presents a dendrogram for the 31 cases classified by ER and PR status using all 29,000 genes examined. A review of these figures and associated dendrograms shows clustering of cases to be more strongly driven by PR rather than ER status. When more formally examined by hormone receptor positivity, upregulation or down-regulation was noted to be statistically significantly related to PR status for a number of genes (Table 1). In the comparison between ER+ and ER- lesions, no genes seemed to reach statistical significance.

of staining, slides were counterstained with hematoxylin, dehydrated through ethanol and xylene, and coverslipped with Permount.

⁶ http://www.r-project.org

⁷ http://www.bioconductor.org

No.	Gene	Chromosome	FDR	Gene name	Function*
Up-r	egulated				
1	SLC24A3	20p13	0.023	solute carrier family 24 (sodium/ potassium/calcium exchanger), member 3	Encodes plasma membrane sodium/calcium exchangers.
2	SERPINB1	6p25	0.032	serpin peptidase inhibitor	Stops, prevents, or reduces the activity of serine-type endopeptidases.
3	FLJ10781	19q13.32	0.032	hypothetical protein FLJ10781	
4 5	KIAA1109 SNRPD3	4q27 22q11.23	0.032	small nuclear ribonucleoprotein D3 polypeptide 18 kDa	Aspartic-type endopeptidase activity. These proteins associate with RNA to form core domain of the ribonucleoprotein particles involved in a variety of RNA processing events.
6 7	PEXIIA NDUFA6	15q26.1 22q13.2-q13.31	0.032 0.032	peroxisomal biogenesis factor 11A NADH dehydrogenase (ubiquinone) 1α subcomplex, 6, 14 kDa	Protein assists with catalysis of the reaction: NADH + H^+ + ubiquinone = NAD ⁺ + ubiquinol.
8 9	LOC401152 FLJ10847	4q26 17p11.2	0.032 0.037	HCV F-transactivated protein 1 hypothetical protein FLJ10847	This gene is located within the Smith-Magenis syndrome region on chromosome 17. It encodes a protein of unknown function.
10	FAT4	4q28.1	0.045	FAT tumor suppressor homologue 4 (<i>Drosophila</i>)	
Dow	n-regulated				
1	PDGFRB	5q31-q32	0.023	platelet-derived growth factor receptor, β polypeptide	Encodes cell-surface tyrosine kinase receptor for members of the platelet-derived growth factor family. The growth factors are mitogens for cells of mesenchymal origin.
2 3	EGFL5 MAP4K4	9q32-q33.3 2q11.2-q12	0.032 0.032	EGF-like domain, multiple 5 mitogen-activated protein kinase	Encodes member of the serine/threonine protein
4	BCL2	18q21.3	0.032	kinase kinase kinase 4 B-cell CLL/lymphoma 2	kinase family. Encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes. Constitutive expression of BCL2 is thought to be the cause of follicular lymphoma.
5	SOX4	6p22.3	0.032	SRY (sex determining region Y) box 4	This intronless gene encodes a member of the SOX (SRY-related HMG-box) family of transcription factors involved in the regulation of embryonic development and in the determination of the cell fate. The encoded protein may act as a transcriptional regulator and may function in the apoptosis pathway leading to cell death as well as to tumorigenesis.
6	BACE2	21q22.3	0.032	β-site amyloid precursor protein-cleaving enzyme 2	This gene localizes to the "Down critical region" of chromosome 21. The encoded protein, a member of the peptidase A1 protein family, is a type I integral membrane glycoprotein and aspartic protease.
7	NLN	5q12.3	0.032	neurolysin (metallopeptidase M3 family)	
8 9	HEGI FMNL3	3q21.2 12q13.12	0.032 0.032	HEG nomologue 1 (zebratish) formin-like 3	The protein encoded by this gene contains a formin
10	TM4SF1	3q21-q25	0.036	transmembrane 4 L six family member 1	Encodes cell-surface proteins that mediate signal transduction events that play a role in the regulation of cell development activation, growth, and motility. This encoded protein is a cell-surface antigen and is highly expressed in different carcinomas.
11 12	EDNRA FSTL1	4 3q13.33	0.036 0.036	endothelin receptor type A follistatin-like 1	Encodes a protein with similarity to follistatin, an activin-binding protein.

Table 1. Genes identified as being significantly up-regulated or down-regulated in meningiomas with PR versus those without PR

	Gene	Chromosome	FDR	Gene name	Function*
13 7	TANC	2q24.1-q24.2	0.036	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1	
14 A	ACSL4	Xq22.3-q23	0.045	acyl-CoA synthetase long-chain family member 4	Encodes isozyme of the long-chain fatty-acid-CoA ligase family, and plays a key role in lipid biosynthesis and fatty acid degradation.

Table 1. Genes identified as being significantly up-regulated or down-regulated in meningiomas with PR versus those without PR (Cont'd)

*Entrez-Gene. Available from: http://www.ncbi.nlm.nih.gov/entrez (accessed January 20, 2007).

The overall distribution of genes up-regulated and downregulated by PR status and by chromosomal arm is presented using a FDR cutoff point of 0.10 in Fig. 4. Genes on the long arm of chromosome 22 (22q) are the most frequently noted to have expression variation, with significant up-regulation in PR+ versus PR- lesions found for 19 genes. This is of interest given that the most frequent chromosomal abnormality identified in patients with meningioma involves the entire loss of chromosome 22 or deletion of the long arm of this chromosome. Other areas of note include the long arms of chromosomes 2 and 3, which show significant down-regulation for PR+ versus PR- lesions, as well as chromosome 4. Of note, 10 genes from chromosome 1, many centered about 1q24, are up-regulated but only if a FDR of 0.10 is used (none are significant if a FDR of 0.05 is used). Little change in regulation by PR status is seen for chromosomes 9 and 14 or other sites of chromosomal abnormalities frequently described in meningiomas, particularly those noted to have clinically progressed.

Specific genes identified as being significantly up-regulated or down-regulated in meningioma by PR status as well as their currently understood function are presented in Table 1. When a false-positive discovery rate (FDR) of 0.05 is used, two genes located on 22q (SNRPD3 at 22q11.23 and NDUFA6 at 22q13.2) show significant overexpression for PR+ versus PR- cases. Of note, all 19 genes identified at a FDR of 0.10 from 22q (including XRCC6 at 22q13.31, a gene involved in the DNA repair pathway) tightly flank the region that includes the NF2 gene (22q12), long associated with meningioma development. Although PR status and grade are frequently correlated in the literature, with PR+ lesions seen more frequently in low-grade meningiomas, this did not explain the association with expression of genes on chromosome 22 and PR status in these data (Fisher's exact test, P = 0.44). Genes located on chromosome 4 are also noted to be significantly up-regulated in PR+ lesions, including three in the same region of the long arm of chromosome 4 (LOC4011 at 4q26, KIAA110 at 4q27, and FAT4 at 4q28.1) at a FDR of 0.05 and 10 genes at a FDR of 0.10. The majority of these genes are located at 4q21-q32. Additional genes that are significantly up-regulated for PR+ lesions at a FDR of 0.05 are located on chromosomal areas 6p, 15q, 17p, 19q, and 20p.

A number of genes were also noted to be significantly down-regulated in PR+ versus PR- lesions. The most frequently reported area of interest is chromosome 2 with two genes underexpressed at a FDR of 0.05 (*MAP4K4* at 2q11 and *TANC* at 2q24) and 15 genes at a FDR of 0.10 (Fig. 2), the majority also placed at the long arm of chromosome 2. At a FDR of 0.05, three genes on the long arm of

chromosome 3 are notable for decreased expression in PR+ lesions (*HEG1* at 3q21.2, *TM4SF1* at 3q21, and *FSTL1* at 3q13) as are genes on the long arms of chromosomes 5, 9, 12, 18, 21, and X. At a FDR of 0.10, 13 genes are down-regulated on chromosome 5 as are 10 genes on both chromosomes 6 and 12. A histogram of the expression levels for the 31 cases by gene and PR status for the top four differentially expressed genes (both up-regulated and down-regulated) is presented in Fig. 5.

Pathway analysis was done on the data (all genes) and although no single pathway or group of genes was clearly identified, a number of pathways involved in the production of collagen and the extracellular matrix were noted to have numerous genes differentially expressed by PR status with a higher proportion of genes upregulated in PR+ lesions than in PR- lesions.

Discussion

The genetic classification of meningiomas remains limited despite the fact that meningiomas were among the first solid neoplasms studied by cytogenetic analyses. Loss of chromosome 22 was reported in meningioma as early as 1967 and is seen in up to 70% of cases (33, 34). The majority of meningiomas with loss on this chromosome have mutations in the NF2 gene located on 22q. Mutation or loss of this gene seems to represent an early genetic event in the development of many meningiomas because it is frequently the predominant genetic abnormality in grade 1 and is found in all grades of meningioma (33). Our data reveal evidence of overexpression of a number of genes located on the long arm of chromosome 22 for cases with positive PR relative to those without evidence of such receptors. Of note, these genes flank the region that includes the NF2 gene (22q12) and may suggest a possible loss of this region in tumors that are PR-. It is also interesting that, in addition to NF2, a number of genes involved in steroid hormone metabolism, including GSTT1 at 22q11.23 and COMT at 22q11.21, are located in this region (although no probes for these genes were present on the chip used). We noted one gene important in doublestrand DNA repair, XRCC6 located at 22q13, to be overexpressed in PR+ lesions relative to PR- lesions. These findings are of interest given the fact that the two most important epidemiologic risk factors identified to date for meningioma are hormones and ionizing radiation (1), with loss of this region potentially associated with an inability to process such exposures. Our finding of an association between PR status and chromosome 22q regulation concurs with that of a recent study designed to compare cytogenetic abnormalities in 154 meningioma specimens by ER and PR status (7). In that report, an increased rate of chromosome 22 abnormalities (primarily loss or monosomy) was appreciated in receptor-negative (ER-/PR-) and ER+ tumors versus PR+ lesions. Although our results are not directly comparable given our relatively small sample size and hence the inability to stratify tumor specimens by both PR and ER status simultaneously, their agreement with this previous study may suggest differing genetic pathways of initiation or progression that are more and less likely to include loss or deletion of the long arm of chromosome 22 as an important step and that may be clinically indicated by measurement of PR status.

After chromosome 22, the genes most frequently reported to have alterations in sporadic meningioma specimens are located on chromosomes 1p, 9p, and 14q, particularly in grade 2 and 3 meningiomas. A recently reported significant association between the DNA repair gene RAD54L 2290 C/T polymorphism and meningioma risk is of great interest given its location on 1p and its role in the DNA repair pathway. Analyses of genetic aberrations in meningioma also indicate that losses of chromosome 9p (which occur in approximately one third of cases and represent the third most frequently reported aberration in meningioma) are associated with loss of both wild-type copies of two genes associated with cell cycle control, CDKN2A (9p21) and CDKN2B (9p21), and that this change is associated with progression to anaplasia in meningiomas. Amplification of 17q has also been noted in anaplastic meningiomas; however, a clear association between this amplification and TP53 mutations has not yet been shown (35).

Little evidence of change in the expression of 1p, 9p, or 14q was appreciated in these data although this may be explained by the relative preponderance of low-grade lesions in this series as well as the small sample size. These results concur with Pravdenkova et al. (7) with respect to chromosomes 1 and 9, but not chromosome 14, with that study finding a higher proportion of chromosome 14 abnormalities involved in PR- than in PR+ tumors.

Several additional chromosomal areas showed differential expression of a number of genes in these data, including upregulation on 4q and 1q. Previous studies have shown frequent changes on 1q primarily in grade 2 and 3 meningiomas with gains or amplifications predominating (33, 36), although no specific proto-oncogene has been localized to this area. Few changes have previously been reported for 4q in meningioma lesions although deletions in this area have been reported for other tumors including breast (37) and hepatocellular carcinoma; little evidence for a tumor suppressor gene located in this area has been found to date.

The genes for the ER and PR expression are located on 6q25.1 and 11q22-q23, respectively. In our data, we noted no significant expression changes in genes from these regions by receptor status with the exception of the gene *CCDC82* (the function of which is not well described) located at 11q21, which was found to be down-regulated at a FDR probability of 0.09. This concurs with a recent study by Pravdenkova et al. (7) that found no significant genetic changes in chromosomes 6 and 11 when meningioma specimens were stratified by ER and PR status.



Figure 4. Number of genes up-regulated (*top*) and down-regulated (*bottom*) in PR+ versus PR- tumors presented by chromosome number (*horizontal axis*) and chromosome arm (*vertical axis*).



Figure 5. Histograms of the top four genes up-regulated (top row) and down-regulated (bottom row) by PR status.

We attempted to define gene pathways for which PR or ER expression differed. Several pathways that included a number of genes with significantly different PR expression were noted, most of which were involved in the production of collagen and the extracellular matrix. This is of interest given the fact that meningiomas include collagen fibers to varying degrees with fibrous tumors containing higher amounts of collagen, mixed or transitional tumors containing an intermediate amount of collagen, and menigothelial tumors containing smaller amounts. The suggestion that genes in such a pathway may be differentially overexpressed by PR expression level, and hence possibly related to meningioma development, warrants further investigation and again raises intriguing questions about whether anti-progesterones may, in some instances, be used to block the activity of collagen genes and possible tumor growth in meningioma.

Limitations to our study include the fact that specimens are drawn from a hospital-based series of cases rather than from a population-based series. In addition, tissue was not available on all cases over the time period of study with a tendency for cases with larger tumor specimens to be included given the greater availability of tissue. Our small sample size precluded extensive analyses stratified concurrently by ER and PR status as well as by grade, likely an important inclusion given the reported associations between grade and receptor status. It is also clear that these analyses may benefit from stratification by sex given the recent findings of a statistically significant difference in the proportion of chromosomal abnormalities between tumors collected from men versus women, especially on chromosomes 7 and 14. As seen in these data, meningiomas show a low rate of ER positivity but higher rates of PR; this, in part, can make statistical detection of an effect difficult as little variation exists for the ER status. With respect to the use of FDRs to define statistical significance, we note that the computation of q values (FDR) based on the P values can be misleading when there is a

nonstandard distribution of P values due to the presence of confounding variables; however, that does not seem to be the case in these data.

As highlighted by previous cytogenetic and gene expression studies (33, 36), our data confirm that meningioma tumors are quite heterogeneous with complex karyotypes expressed in the majority of lesions. Although the relationship between expression profiles and hormone receptor status is clearly multifaceted, it is of note that the relative expression of a number of genes varied by PR expression level but did not seem to vary significantly by ER level. The fact that PR status seemed to be associated with changes near the NF2 gene on 22q (mutations in which are identified as being an important initial event in meningioma development) continues to suggest that hormones are likely to play an important role in either the development or progression of some meningiomas and/or that PR status may be an important clinically measurable indicator variable of that role. Results such as these highlight the potential clinical value of accurate knowledge of PR status in meningiomas, particularly given the reports that the expression of PR (either alone or in concert with other predictive variables such as mitotic index) signals a more favorable clinical prognosis (7). The specific relationships between PR status and expression of the genes or chromosomal areas noted in this analysis remain unclear and will require further study in a larger sample collected from a population-based data set.

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