molecular epidemiologic studies. DNA bases are particularly susceptible to oxidation mediated by reactive oxygen species, which can be produced as a consequence of ionizing radiation or environmental exposure to transition metals, chemical oxidants, and free radicals. Reactive oxygen species have been linked to the initiation and progression of cancer.[29] BER plays an important role in preventing mutations associated with a common product of oxidative damage to DNA, 8-oxoguanine. X-Ray Repair Cross-Complementing Group 1 (*XRCC1*), located on 19q13.2, is a polymorphic BER gene that has been the most extensively examined in molecular epidemiologic studies of the risk of various cancers.[30] In the above-mentioned population-based case–control study conducted in 6 areas of San Francisco Bay,[31] a synergistic effect between the *XRCC1 399*Gln allele and tobacco smoking in relation to pancreatic cancer risk was observed, although no significant associations were noted between *XRCC1* genotypes and pancreatic cancer risk. As compared with never-active smokers and passive smokers with the Arg/Arg genotype, the age- and race-adjusted ORs for heavy smokers (≥41 pack-years) with the Gln/Gln or Arg/Gln genotypes were 7.0 (95% CI, 2.4–21) in women and 2.4 (1.1–5.0) in men. The interaction suggests that *XRCC1* Arg399Gln and BER capacity are important in susceptibility to smoking-induced pancreatic cancer. However, these findings need to be confirmed in other studies, as the number of study subjects was small in the analysis exploring gene–environment interaction.

The 8-oxoguanine DNA glycosylase (*OGG1*) gene is another BER gene that removes oxidative DNA lesions.[28] *OGG1* has been associated with altered risk of human cancers. In data from the hospital case–control study conducted at MD Anderson Center in the United States, Li et al noted significantly reduced overall survival in patients with the *OGG1* C315G (rs1052133) GG homozygous variant genotype.[32] Furthermore, they reported a weak interaction of the *OGG1* C315G CC/CG genotype with diabetes in pancreatic cancer. These findings suggest that the CC/CG genotype, combined with environmental exposure, confers increased susceptibility to pancreatic cancer.

Li et al also examined associations of pancreatic cancer with selected DNA repair polymorphisms in other types of DNA repair pathways, including *XRCC2*, *XRCC3*,[33] *RAD54L*, and *RecQ1* in the recombination repair pathway,[34] and the xeroderma pigmentosum group D (*XPD*) in the NER pathway.[35] They found that variant alleles of *XRCC2* R188H and *XRCC3* A17893G were associated with significantly reduced survival in pancreatic cancer patients and that *XRCC2* Arg188His polymorphisms may be genetic modifiers for smoking-related pancreatic cancer.

Overall, evidence from a small number of molecular epidemiologic studies supports a role for genetic variability in DNA repair in the risk for pancreatic cancer. Due to small sample sizes and heterogeneous study designs, however, the results are inconclusive and require confirmation. Because hundreds of genetic polymorphisms may be involved in maintaining genomic integrity, additional studies with large sample sizes are needed to elucidate multiple sequence variants in a gene or multiple genes within an entire pathway.

Folate intake and polymorphisms in folate-metabolizing genes

Folate is a water-soluble B vitamin abundant in green leafy vegetables, citrus fruit, legumes, and cereals. Substantial evidence from epidemiologic and laboratory research supports a role for folate in carcinogenesis.[36,37] Epidemiologic studies have consistently shown an inverse association between folate intake and pancreatic cancer risk. Based on a meta-analysis in which data from 4 cohort studies and 1 case–control study were analyzed, individuals with the highest folate intake had a 51% lower risk than those with the lowest folate intake.[38] Mechanistic studies have elucidated 2 major underlying mechanisms that may be involved. Folate deficiency may induce misincorporation of uracil into DNA, leading to chromosomal breaks and mutations. In addition, folate deficiency may cause aberrant DNA methylation, resulting in altered expression of critical proto-oncogenes and tumor suppressor genes.[39] Moreover, functional polymorphisms in folate-metabolizing genes may confer susceptibility to cancer. Among the several polymorphisms in the folate metabolic pathway, polymorphisms in the 5-10 methylenetetrahydrofolate reductase (*MTHFR*) gene are the most extensively studied. A central enzyme in folate metabolism, MTHFR irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant form of folate in systemic circulation. Thus, *MTHFR* acts as a critical junction in folate metabolism by directing folate metabolites toward the DNA methylation pathway and away from the DNA synthesis pathway. Two common functional polymorphisms of the *MTHFR* gene, C677T and A1298C, have been identified.[39] Regarding C677T, the TT genotype (variant type) has been shown to have 35% lower enzyme activity than the CC genotype (wild type).[39] As for A1298C, homozygotes (CC) have approximately 60% of normal *MTHFR* activity.

Since 2005, five studies have reported an association between the *MTHFR* C677T genotype and pancreatic cancer risk, but the results were not consistent[9,40–43] (Table 2). The TT genotype was associated with significantly increased risk for pancreatic cancer in 2 case–control studies carried out in the United States[9] and China[40]: the ORs were 2.14 (95% CI, 1.14–4.01) and 5.12 (2.94–9.10), respectively. Notably, a significant interaction between TT genotype and smoking in pancreatic cancer risk was also observed. In these 2 studies, heavy smokers with the TT genotype had an approximately 7-fold increased risk as compared with nonsmokers with the CC genotype.[9,40] In contrast, 2 Japanese case–control studies reported no increased risk associated with the TT

Table 2. Summary of findings from case–control studies of genetic polymorphisms in folate-metabolizing genes and their interactions with environmental factors and pancreatic cancer risk

Study and year	Study population	No. of cases	No. of controls	Genetic polymorphisms	Main effects of polymorphisms[a]	Gene–environment interaction[a]
Li et al, 2005 (Ref 9)	Non-Hispanic US whites	347	348	*MTHFR* C677T, A1298C	Significant effect for C677T: CT, 0.90 (0.63–1.27), TT, 2.14 (1.14–4.01); no association for A1298C	Heavy smokers with TT vs never smokers with CC/CT: 6.83 (1.91–24.38) Heavy alcohol drinkers with TT vs nondrinkers with CC/CT: 4.23 (0.88–20.3)
Wang et al, 2005 (Ref 40)	Chinese	163	337	*MTHFR* C677T, A1298C, TS	Significant effect for C677T: CT, 2.60 (1.61–4.29), TT, 5.12 (2.94–9.10); no association for A1298C	Heavy smokers with TT vs never smokers with CC/CT: 6.69 (3.39–13.63) Alcohol drinkers with TT/CT vs nondrinkers with CC: 4.39 (2.25–8.78)
Matsubayashi et al, 2005 (Ref 41)	Americans	303	305	*MTHFR* C677T, A1298C	No association for C677T: CT, 0.79 (0.56–1.11), TT, 1.10 (0.67–1.82)	No significant interaction with smoking
Suzuki et al, 2008 (Ref 42)	Japanese	157	785	*MTHFR* C677T, MTR A2756G, TS variable number of tandem repeat	No association for C677T: CT, 0.98 (0.65–1.47), TT, 0.75 (0.41–1.35)	No significant interaction with alcohol drinking
Ohnami et al, 2008 (Ref 43)	Japanese	198	182	*MTHFRC677T*, MTRR (rs1801394, rs162049, rs10380)	No association for C677T, but MTRR polymorphisms associated with increased risk	No association

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; OR, odds ratio.
[a]Values are odds ratios (95% confidence interval).

Table 3. Associations of genetic polymorphisms and pancreatic cancer risk that require assessment in future studies

Candidate genes	Selected polymorphisms	Potential interactions with environmental factors	Circulating biomarker
Vitamin D signaling	rs11574143	Sun exposure, diet	Plasma 25-hydroxyvitamin D
Melatonin receptors and clock genes	*MTNR1B*, rs10830963, rs11133373 in *CLOCK*	Diabetes	Plasma or urinary melatonin (6-sulfatoxymelatonin)
Insulin, IGF gene	IGF1 haplotype and the IGF2 Ex4 -233 C>T TT genotype	Diabetes, obesity	Plasma or serum IGF
TGF-β signaling	*TGFBR1*6A*	Diabetes	Plasma or serum TGF-β
Infection-related gene polymorphisms	COX-2 polymorphisms	N/A	N/A
ABO gene	rs505922	N/A	N/A
Genes in chromosome 13q22.1	Novel polymorphisms to be identified	N/A	N/A

Abbreviations: IGF, insulin-like growth factor; TGF-β, transforming growth factor-β; MTNR1B, melatonin receptor 1B; COX-2, cyclooxygenase-2.
N/A: not applicable.

genotype.[41,42] In one of these studies, the OR for pancreatic cancer in individuals with the TT genotype was 0.75 (0.41–1.35), but the association was statistically insignificant.[42] Due to the small number of studies and the wide heterogeneity in results, a summary OR could not be calculated in a meta-analysis of the *MTHFR* C677T genotype and pancreatic cancer risk, as only 3 published studies were included.[38] Inadequate sample size and different criteria for control selection might have contributed to the inconsistent results reported so far. As for the A1298C genotype, evidence is insufficient to draw a conclusion: 2 studies showed no important effects on pancreatic cancer, and one study suggested a 1.8-fold increased risk in subjects with the CC genotype.[38]

Given the strong interaction of the *MTHFR* genotype with environmental factors such as smoking and alcohol consumption, it is important to unravel their complex relationships in additional large, adequately powered studies. Furthermore, because the balance between the use of methylenetetrahydrofolate for DNA synthesis rather than for methionine synthesis might depend on the presence of the 677T variant of *MTHFR* and nutritional folate status, studies targeting populations with folate deficiency in developing countries may provide valuable information.

Alcohol consumption and polymorphisms in alcohol-metabolizing enzymes

Although the majority of prospective cohort studies found no significant increase in the risk of pancreatic cancer with moderate to high levels of alcohol intake in a general population,[7] some evidence suggests that excessive drinking may increase risk in population subsets.[44]

Ethanol is mainly metabolized to acetaldehyde by alcohol dehydrogenase enzymes and further oxidized to acetate by acetaldehyde dehydrogenase. Acetaldehyde has been shown to have carcinogenic effects in experimental studies and is the main mechanism to explain alcohol-induced carcinogenesis.[45] Variations in the production and/or oxidation of acetaldehyde among individuals are caused by single-nucleotide polymorphisms (SNPs) of *ADH1B*, *ADH1CI*, and *ALDH2*.[45] In particular, people homozygous for *ALDH2**2 display flush syndrome, which is characterized by nausea, vomiting, and facial flushing after ingestion of a small amount of alcohol.

Very few studies have addressed the role of polymorphisms in alcohol-metabolizing enzymes and pancreatic cancer risk. A recent case–control study involving 160 pancreatic cancer patients and 800 age- and sex-matched controls in Japan found that alcohol consumption was associated with increased risk in individuals with the *ALDH2* Lys+ allele or *ADH1B* His/His or *ADH1C* Arg/Arg genotypes, but not in those with the *ALDH2* Glu/Glu genotype or *ADH1B* Arg or *ADH1C* Gln alleles.[46] This suggests that the risk of pancreatic cancer is associated with the combined effect of alcohol consumption and certain polymorphisms in alcohol-metabolizing enzymes.

Because the metabolism of alcohol and acetaldehyde is strongly influenced by alcohol-metabolizing enzymes, future molecular epidemiologic studies need to examine the effect of these polymorphisms on pancreatic cancer risk while accounting for alcohol consumption.

Vitamin D and polymorphisms in vitamin D pathway genes

Humans get vitamin D mainly from exposure to sunlight or their diet. Vitamin D is metabolized in the liver to 25-hydroxyvitamin D [25(OH)D], which is further metabolized in the kidneys by the enzyme 25-hydroxivitamin D-1α-hydroxylase (CYP27B1) to its active form, 1,25-dihydroxyvitamin D.[47] Vitamin D receptor, a crucial mediator of the cellular effects of vitamin D, is present in a variety of cell types, including pancreatic beta cells.[48] Experimental evidence shows that vitamin D receptor interacts with other cell-signaling pathways to influence cancer development.[49] Ecologic studies have linked sun exposure to lower pancreatic cancer mortality.[50] Individuals with higher circulating 25(OH)D levels have been found to have decreased risks of breast, colorectal, and prostate cancer in numerous prospective studies.[51] Given the collective evidence from epidemiologic and experimental studies, it is plausible that high vitamin D levels may be associated with a lower risk of pancreatic cancer. However, the role of vitamin D in the development of pancreatic cancer remains unclear due to the small number of studies.

High vitamin D exposure is hypothesized to decrease cancer risk, possibly through genomic effects modulated by the vitamin D receptor, and by autocrine/paracrine metabolism of the vitamin D receptor's ligand, 1α,25-(OH)2-vitamin D3.[49] Recently, an increasing number of studies have examined polymorphisms in vitamin D receptor and selected genes in the vitamin D pathway in relation to colorectal, breast, and prostate cancer risk.[48] However, there is currently no strong, consistent epidemiologic evidence for a substantial influence of any single variant in vitamin D pathway genes on cancer risk. The association of pancreatic cancer with serum vitamin 25(OH)D levels and polymorphic variants in genes encoding for enzymes that synthesize, carry, and degrade vitamin D is an important research subject for future studies.

Circadian disruption, melatonin, and genetic variations in clock genes

To date, no studies have examined the association between circadian disruption and pancreatic cancer risk in humans. Experimental data, however, have shown that disruption of circadian rhythms in mice is associated with accelerated growth of pancreatic cancer.[52] Although the findings are not entirely consistent, epidemiologic studies have indicated that shift work is significantly associated with increased risks of breast, colorectal, and prostate cancer.[53] On the basis of considerable evidence from animal studies and limited evidence from epidemiologic studies, the working group of IARC concluded in 2007 that "shift-work that involves circadian disruption is probably carcinogenic to humans."[54] The principal mechanism involves melatonin, a neurohormone that regulates the circadian rhythm.[55] Three recent GWAS have shown that the common variant *MTNR1B* (melatonin receptor 1B) is associated with insulin and glucose concentrations.[56–58] The melatonin receptor MT1 is highly expressed in pancreatic islet cells, and the expression of *MTNR1B* has been confirmed in both islets and sorted beta cells.[59] Given the close relationship of hyperinsulinemia and diabetes with pancreatic cancer risk, it might prove interesting to examine the risk genotypes of *MTNR1B* and their interactions with diabetes and other environmental factors in pancreatic cancer development.

In addition to melatonin, circadian rhythms are controlled and maintained by several circadian genes via transcription–translation feedback loops that include positive activators, such as Clock, neuronal PAS domain protein 2 (*NPAS2*), cryptochrome 1 (*CRY1*) and *CRY2*, and period 1 (*PER1*), *PER2*, and *PER3*.[60] To test the hypothesis that genetic variations in these genes may confer susceptibility to prostate cancer, Zhu et al genotyped a total of 41 tagging and amino acid-altering SNPs in 10 circadian genes in a population-based case–control study of white men and found

that *NPAS2* showed the most robust association with prostate cancer risk.[61] No studies, however, have examined genetic polymorphisms in clock genes and pancreatic cancer risk.

Because of the important role of melatonin and circadian genes in maintaining circadian rhythm, future studies may address genetic variations in these genes and the risk of pancreatic cancer.

Insulin and insulin-like growth factor gene polymorphisms

Obesity and type II diabetes are well established risk factors for pancreatic cancer, especially in developed countries. Elevated levels of insulin and insulin-like growth factors (IGFs), such as IGF-I, are important mechanisms underlying the association between obesity, diabetes, and pancreatic cancer.[62] Insulin, IGF-1, and the insulin receptor-related receptor can form functional hybrids.[63] IGF1 and IGF1 receptors are highly expressed in pancreatic cancer cells, and IGF2 imprinting is disrupted in many tumors.[64]

Despite strong experimental evidence indicating that IGFs play an important role in carcinogenesis—including the regulation of cell proliferation, differentiation, and apoptosis—the results of epidemiologic studies examining IGFs in relation to cancer risk are less persuasive. Using data from a nested case–control study in the Japan Collaborative Cohort (JACC) Study, we found a positive association between baseline IGF-1 levels and the risk of pancreatic cancer mortality in apparently healthy Japanese.[65] However, no significant associations were observed in other studies.[66] Only 1 study has examined the association between genetic polymorphisms in IGF genes and pancreatic cancer risk.[67] Of 6 SNPs of IGF1 and IGF2 that were examined in a case–control study by Suzuki et al, the IGF1 haplotype and the IGF2 Ex4 -233 C>T TT genotype were significantly associated with decreased risk of pancreatic cancer, which suggests that polymorphic variants of the IGF genes may serve as a susceptibility factor for pancreatic cancer. Future studies are warranted to explore polymorphisms in IGF gene pathways and their interaction with obesity and physical activity in pancreatic cancer risk.

Transforming growth factor-β (TGF-β) and polymorphisms in the TGF-β pathway

TGF-β regulates tumor initiation, progression, and metastasis via its signaling pathway involving membrane receptors and SMAD transcription factors.[68] The dual role of TGF-β in cancer, both as a tumor suppressor and tumor promoter, has been well defined.[69] Several lines of evidence demonstrate that pancreatic cancer is clearly linked to TGF-β.[70] In particular, SMAD4, a component of the TGF-β pathway, is mutated in approximately 50% of pancreatic cancers.[71]

Because of the presence of plausible mechanisms, a study of the associations of polymorphisms in TGF-β pathway with pancreatic cancer risk should prove interesting. *TGFBR1*6A* is emerging as a high frequency, low-penetrance tumor susceptibility allele that confers susceptibility to breast, ovarian, and colorectal cancer. A meta-analysis of 7 case–control studies of *TGFBR1*6A* and various cancer types combined showed that *TGFBR1*6A* carriers had a 26% increased risk.[72] The role of *TGFBR1*6A* in pancreatic cancer remains unclear and is a subject of future study.

Inflammation and infection-related gene polymorphisms

Inflammation has been implicated in pancreatic carcinogenesis. Cyclooxygenase-2 (COX2) is a key enzyme involved in biologic processes including inflammation, immune function, and cell proliferation.[73] The overexpression of this enzyme has been shown in pancreatic cancer.[74] However, there have been few studies addressing inflammation-related genetic polymorphisms and pancreatic cancer risk. Zhao et al showed that functional *COX-2* polymorphisms are associated with susceptibility to pancreatic cancer.[75] In another case–control study, 3 infection-related polymorphisms (*TNF-A, RANTE5, and CCR5*) were examined, but no significant effects were found.[76]

Helicobacter pylori infection induces chronic inflammation and has been established as a risk factor for gastric cancer. It remains controversial, however, whether *H. pylori* infection plays a role in pancreatic cancer development.[77] Epidemiologic studies examining this issue have produced mixed results. The hypothesis has been proposed that polymorphisms in genes involved in inflammatory response, such as *IL-1A, IL-1B, IL-6, IL-8*, may help explain why only a subset of individuals infected with *H. pylori* develops gastric cancer. Similarly, it is important to comprehensively analyze the effects of these polymorphisms on pancreatic cancer risk.

SNPs indentified by genome-wide association studies

Genome-wide association studies (GWAS) have been proven to be a valuable tool for identifying common alleles that influence disease risk.[78] Fast-evolving sequencing technology allows researchers to scan across the genome in a large set of cases and controls to identify new associations that link certain regions to disease risk.

The first GWAS for pancreatic cancer, published in *Nature Genetics* in August 2009, identified common risk variants that map to the first intron of the *ABO* gene on chromosome 9q34.2 (SNP rs505922).[11] This finding implies that people with blood group O may have a lower risk than those with groups A or B.

Base on DNA collected from nearly 4000 patients in 13 different studies, a second GWAS for pancreatic cancer has, for the first time, identified pancreatic cancer susceptibility loci on 3 chromosomes—13q22.1, 1q32.1, and 5p15.33.[12] Of the 3 regions, the locus on 13q22.1 appears to be specific for pancreatic cancer.

Findings from the 2 GWAS of pancreatic cancer have provided new insight into pancreatic cancer etiology; however, they need to be replicated in other large studies. Furthermore, follow-up studies after the GWAS must address whether SNPs discovered by GWAS represent functional variants or simply tag true variants located in the same haplotype.

Mitochondrial genetic polymorphisms

Mitochondria play an important role in cellular energy metabolism, free radical generation, and apoptosis. Mitochondrial DNA mutations can initiate a cascade of events leading to persistent oxidative stress, a condition that probably favors tumor development.[80] Although previous studies have examined the association between mitochondrial genetic polymorphisms and pancreatic cancer,[81–83] the results were inconsistent. While a mitochondrial SNP in the 16519 mitochondrial DNA nucleotide was found to be associated with worse prognosis,[81] this positive association was not replicated in a recent study involving 990 pancreatic cancer patients.[82] A recent large case–control study comprised 955 participants with primary pancreatic adenocarcinoma and 1102 control subjects and examined 24 mitochondrial SNPs and 11 common haplogroups, none of which was significantly associated with pancreatic cancer risk.[83] Their results did not support the significant involvement of mitochondrial SNPs or haplogroups in the development of pancreatic cancer. Because of the important role of mitochondrial DNA in cancer, further investigations of mitochondrial genetic variations are necessary to provide insight into the etiology of pancreatic cancer.

DISCUSSION

Molecular epidemiologic studies examining the associations between polymorphisms in several gene pathways and pancreatic cancer risk have produced mixed results. Overall, individual polymorphisms did not seem to confer marked susceptibility; however, some studies implicated interactions of polymorphisms in carcinogen-metabolizing genes, DNA repair genes, and folate-metabolizing genes with smoking, diet, and obesity. The principal weakness of these studies is small sample size; thus, it is difficult to detect statistically significant gene–gene or gene–environment interactions. Because of this, no functional variants reported so far have been used to predict pancreatic cancer risk in the clinical setting. Another critical challenge is that the measurement of environmental influence in epidemiologic studies must be improved to better define gene–environment interaction.

Two recent GWAS of pancreatic cancer have provided intriguing results that need to be confirmed in additional studies. With the decreasing cost of genotype sequencing, we expect that future GWAS will unravel causal variants with significant effects on pancreatic cancer. Hopefully, disease susceptibility variants will be discovered from GWAS, and the interactions of these variants with environmental factors will be more frequently confirmed in molecular epidemiologic studies. Since we have yet to discover rare variants that greatly increase the risk of pancreatic cancer, perhaps, as is the case with other complex diseases, common low-risk variants in different genes act collectively to confer susceptibility to pancreatic cancer in individuals who have repeated environmental exposures, such as smoking and intake of red meat. A recent study provided critical evidence to support this notion by demonstrating that pancreatic cancer results from genetic alterations of a large number of genes that function through 12 pathways and processes,[79] including TGF-β signaling and DNA damage control, which were discussed in this review.

What is the future direction for research on the etiology of pancreatic cancer? First, we believe that unraveling the functional SNP variants in a number of identified gene pathways, combined with novel variants identified in GWAS, is essential in deepening our understanding of pancreatic cancer risk. To achieve this goal, the complex gene–gene and gene–environmental interactions must be clarified in a rigorously designed molecular epidemiologic study with a large sample size. Second, in addition to SNPs, there is increasing recognition of the role of genetic variations—such as DNA copy number variations and variable-number tandem repeats—in cancer predisposition.[84] High-resolution SNP arrays have made it possible to identify copy number variations. Moreover, there have been studies linking copy number variations and variable-number tandem repeats to pancreatic cancer risk.[85,86] Elucidating these associations is an important goal for future research.

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ORIGINAL ARTICLE

Relationship of sFas with metabolic risk factors and their clusters

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ABSTRACT

Background Metabolic risk factors are known to cause atherosclerosis through inflammation. In the process of inflammation, soluble Fas (sFas) may interfere with the apoptotic pathway and contribute to dysregulated inflammation. Recent studies suggest sFas as a marker of inflammation in patients with cardiovascular diseases. However, whether a relationship exists between sFas levels and metabolic risk factors among healthy subjects remains unclear.

Materials and methods We measured the serum sFas levels of 876 subjects selected as controls for a nested case–control study within the JACC Study. The adjusted means of the sFas levels were compared according to the presence of overweight/obesity, hypertension, hyperlipidaemia, diabetes and their clusters.

Results sFas level was significantly associated with overweight/obesity (2·42 ng mL^{-1} in overweight/obese men and 2·19 in others) and hyperlipidaemia (2·34 ng mL^{-1} in men with hyperlipidaemia and 2·19 in others) among men, though not with hypertension or diabetes. Moreover, a clear association between sFas levels and the cluster number of metabolic risk factors was observed independently with age, smoking and drinking(2·39, 2·28, 2·24 and 2·11 ng dL^{-1} in men with three to four, two, one and none of the four metabolic risk factors respectively). However, among women, clear associations were not observed between sFas levels and the four metabolic risk factors or their clustering.

Conclusions Serum sFas levels appear to be associated with overweight/obesity, hyperlipidaemia and clusters of metabolic risk factors among men, suggesting that sFas may elevate to down-regulate increased apoptosis in atherogenesis processes.

Keywords Metabolic risk factors, sFas.

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Introduction

Atherosclerosis has recently been recognized as an inflammatory disease linked to an abnormality of oxidation-mediated signals in the vascular system [1]. The inflammatory response plays an important role in the development of atherosclerosis, and epidemiological studies have revealed an association between inflammation and future risk for coronary heart disease [2,3]. The common risk factors for atherosclerosis, such as smoking, obesity, hypertension, hyperlipidaemia and diabetes, induce systemic oxidative stress and increase production of reactive oxygen species (ROS) [4,5]. These ROS initiate several processes involved in atherogenesis, including stimulation of vascular smooth muscle proliferation and migration, apoptosis in the endothelium and oxidation of lipids [5]. In recent studies, soluble Fas (sFas), an isoform of Fas which induces an apoptotic signal with binding to Fas ligand, was suggested to be a marker of inflammation in hyperthyroidism [6], systemic lupus erythematosus [7] and chronic kidney disease [8]. Moreover, increased sFas levels were found to predict future cardiovascular disease in patients with end-stage renal disease [9]. However, it remains unclear whether a relationship exists between sFas levels and metabolic risk factors among the general population.

We had already compared serum sFas levels of cancer mortality cases with their controls in a nested case–control study [10]. The controls we had selected in that study were from a general population but some had a history of hypertension or diabetes, and at the same time, some had anthropometric records at baseline. On the basis of such backgrounds, we investigated the possible association between metabolic risk factors drawn from such anthropometric records and sFas levels using the controls of our previous nested case–control study.

Materials and methods

Study population and serum samples

The study subjects were the controls of a nested case–control study within the Japan Collaborative Cohort (JACC) Study, a large-scale cohort study. Details of the JACC Study have been described elsewhere [11,12]. Briefly, it involved 110 792 subjects, aged 40–79 years at baseline living in 45 municipalities all over Japan. At the baseline, information on lifestyle factors was collected using self-administered questionnaires. In most areas, subjects were recruited at the time of group health check-ups and from some of them, the records of the check-ups were also collected for the study. Additionally, 35% of the cohort participants donated blood samples, and the frozen sera were kept at −80 °C until analyses were performed.

Those who died prior to the end of 1997 or suffered from cancer prior to the end of 1994 were regarded as cases of the nested case–control study. For each case, we randomly selected 3–4 controls, matching for sex, age and residential area. Eventually, 2867 cases and 10 350 controls were chosen. In this study, the subjects were selected from the controls of the nested case–control study. The study design and use of serum were approved by the Ethical Board at Nagoya University School of Medicine, where the central office of the JACC Study was located.

Definition of metabolic risk factors and their clustering

All information on lifestyle factors and medical history was obtained by self-administered questionnaires. In some areas, records were kept from health check-ups (i.e. at baseline) including height, weight, blood pressure and the levels of some serum components. Body mass index (BMI) was calculated based on the examined height and weight (BMI = weight (kg)/height $(m)^2$). We tried to define metabolic risk factors according to the modified criteria of the National Cholesterol Education Program Adult Treatment Panel III (NCEP/ATPIII) [13] as far as possible. Overweight/obesity was defined as a BMI ≥ 25 kg m^{-2}, since waist circumference was not measured in the present study. Hyperlipidaemia was defined as the state in which serum triglycerides ≥ 1·69 mmol L^{-1} (150 mg dL^{-1}) or HDL-cholesterol < 1·03 mmol L^{-1} (40 mg dL^{-1}) for men and < 1·29 mmol L^{-1} (50 mg dL^{-1}) for women. Hypertension was defined as blood pressure of ≥ 130/85 mmHg and/or when subjects reported a medical history of hypertension in their self-administered questionnaires. The subjects were defined as having diabetes when they reported a diabetes medical history since no records on serum glucose were kept. As a result, hyperlipidaemia and hypertension were defined according to the modified criteria of the NCEP/ATPIII [13], whereas the definitions of obesity and diabetes were different. Subsequently, the cluster numbers of these four metabolic risk factors (overweight/obesity, hyperlipidaemia, hypertension and diabetes) that each subject had were compiled. At the time of our baseline survey, it was not common to examine HDL-cholesterol or triglycerides. Only the subjects from six areas had the necessary information to define the metabolic risk factors mentioned above, and the analyses were restricted to these subjects.

Biochemical assays of sera

Serum levels of sFas were measured by enzyme-linked immuno-adsorbent assay (ELISA) in 1999 and 2000, using commercially available kits (MBL Co. Ltd., Nagoya, Japan). All samples were assayed at a single laboratory (SRL Inc., Hachioji, Japan) by trained staff. Assay methods have been described in detail elsewhere [14]. The range of the assay for the serum sFas level was 1·0–10 ng mL^{-1}; the intra- and inter-assay precisions were 2·1–5·5% and 8·2–12·3% respectively. For the present analysis, 928 subjects (532 men and 396 women) were eligible with data on sFas level and information on all metabolic risk factors.

Analytical method

Distributions of some baseline characteristics were compared according to the quartile of sFas levels using the Mantel-Haenszel test. Cut-off points were calculated based on the distribution of the subjects' sFas levels, men and women combined. The means of serum sFas according to the presence of metabolic risk factors and their clusters were adjusted for possible confounding factors using the analysis of covariance. Since the sFas levels had logarithmic distributions, all tests and estimations were conducted using log-transformed levels. Adjusted means of sFas were estimated (i) based on all the subjects, and (ii) based on the subjects excluding those with high sFas concentration (> 10 ng mL^{-1}, $n = 3$) and with a past history of stroke or cardiovascular disease ($n = 49$) as these conditions were suspected to elevate the sFas level. Variables adjusted in multivariate analysis were age group at baseline, area, smoking state (current smoker, ex-smoker, non-smoker and unknown), alcohol consumption (current drinkers, quitters, non-drinkers and unknown) as they were significantly associated with sFas concentration in the present study. All *P*-values were unadjusted

and two-sided, and all statistical analyses were performed using the Statistical Analysis System (SAS 9·1, Cary, NC, USA).

Results

Table 1 shows the distribution of baseline characteristics according to sFas quartiles (≤ 1·8, 1·8–2·1, 2·1–2·4 and > 2·4 ng mL^{-1}). Those with higher sFas levels were statistically older than those with lower levels. Among men, smokers tended to be observed more and drinkers tended to be observed less among higher sFas levels. Though not significant, the men in the top quartile were walking longer and highly educated compared to other men. Similarly, women with higher sFas levels were less likely to eat green leafy vegetables compared to those with lower levels, although education showed the opposite trend to that of men.

Adjusted means of sFas according to the presence of metabolic risk factors and their clusters are shown in Table 2. Overweight/obesity and hyperlipidaemia were statistically associated with increased sFas levels; however, hypertension and diabetes did not show any association with sFas levels among men. Among women, none of these four factors were found to be associated with sFas levels. Clustering numbers of these four metabolic risk factors were clearly statistically associated with sFas levels independent of age, smoking and drinking among men. The adjusted means of the sFas levels increased according to the increase in the cluster numbers, and the top category (presence of 3–4 metabolic risk factors) showed a statistically higher level compared with the lowest category (without any of these risk factors: 2·39 ng mL^{-1} vs. 2·11 ng mL^{-1}). However, no association was observed among women between sFas levels and clustering numbers of four metabolic risk factors, although an increasing trend did exist except in the lowest category. Excluding the subjects with high sFas levels (> 10 ng mL^{-1}) and with a past history of stroke or cardiovascular disease did not alter the result.

Discussion

We found that the sFas level was statistically significantly associated with overweight/obesity and hyperlipidaemia among men, although not with hypertension or a history of diabetes.

Table 1 sFas levels and some baseline characteristics

	sFas level (ng mL^{-1})					
	≤ 1·8	1·8–2·1	2·1–2·4	> 2·4	Total	*P*-value*
Men						
Mean age at baseline†	60·5	61·2	62·3	63·8	61·9	< 0·01
Current cigarette smoker (%)	41·0	43·3	50·5	47·7	45·3	0·01
Current alcohol drinker (%)	79·7	74·4	68·2	60·6	71·0	< 0·001
Walking ≥ 1 h day^{-1} (%)	30·6	27·0	31·2	35·6	30·9	
College or higher education (%)	13·3	13·2	17·8	22·0	16·4	
Eating green vegetables almost daily (%)	28·7	31·6	35·0	30·4	31·1	
Total number	153	133	110	136	532	
Women						
Mean age at baseline†	56·6	61·8	62·5	64·1	61·6	< 0·0001
Current cigarette smoker (%)	5·0	3·7	6·5	3·4	4·7	0·09
Current alcohol drinker (%)	27·1	17·9	15·8	15·7	20·4	
Walking ≥ 1 h day^{-1} (%)	24·1	39·3	36·7	35·3	31·8	
College or higher education (%)	7·4	7·7	4·1	5·0	6·3	
Eating green vegetables almost daily (%)	34·6	40·4	35·4	30·0	35·1	
Total number	145	84	78	89	396	

Each lifestyle factor has missing values, and total is not 100%.
*Adjusted for age groups and residual area.
†Adjusted for residual area.

Table 2 Adjusted means of sFas and metabolic risk factors

	Total subjects						Subjects excluding those with high sFas level or a disease history					
	N	%	Adjusted means of sFas (ng mL^{-1})	95% CI		*P*-value*	*N*	%	Adjusted means of sFas (ng mL^{-1})	95% CI		*P*-value*
Men												
Overweight/obesity												
Absence	395	74·2	2·19	2·06	2·33	< 0·001	368	74·6	2·21	2·09	2·34	< 0·01
Presence	137	25·8	2·42	2·25	2·60		125	25·4	2·40	2·25	2·56	
Hypertension												
Absence	129	24·2	2·24	2·08	2·41	0·71	122	24·7	2·21	2·07	2·35	0·13
Presence	403	75·8	2·26	2·13	2·41		371	75·3	2·29	2·17	2·42	
Hyperlipidaemia												
Absence	336	63·2	2·19	2·06	2·34	0·01	316	64·1	2·22	2·09	2·35	0·03
Presence	196	36·8	2·34	2·19	2·50		177	35·9	2·33	2·20	2·48	
Diabetes												
Absence	488	91·7	2·26	2·13	2·41	0·50	453	91·9	2·27	2·15	2·40	0·73
Presence	44	8·3	2·20	1·98	2·43		40	8·1	2·24	2·05	2·45	
Number of metabolic risk factors												
			Trend P = 0·03						Trend P = 0·01			
0	82	15·4	2·11	1·95	2·29	Ref.	80	16·2	2·13	1·99	2·29	Ref.
1	213	40·0	2·24	2·09	2·40	0·12	198	40·2	2·25	2·11	2·40	0·10
2	148	27·8	2·28	2·13	2·45	0·04	134	27·2	2·28	2·14	2·42	0·06
3–4	89	16·7	2·39	2·21	2·59	< 0·01	81	16·4	2·41	2·25	2·59	< 0·01
Total	532	100·0					493	100·0				
Women												
Overweight/obesity												
Absence	277	69·9	2·17	1·91	2·47	0·67	268	70·0	2·17	1·92	2·45	0·42
Presence	119	30·1	2·20	1·93	2·51		115	30·0	2·22	1·96	2·51	
Hypertension												
Absence	116	29·3	2·19	1·91	2·51	0·87	114	29·8	2·21	1·94	2·51	0·70
Presence	280	70·7	2·18	1·92	2·47		269	70·2	2·18	1·94	2·46	
Hyperlipidaemia												
Absence	194	49·0	2·19	1·92	2·49	0·87	187	48·8	2·18	1·93	2·46	0·78
Presence	202	51·0	2·18	1·92	2·47		196	51·2	2·19	1·94	2·47	
Diabetes												
Absence	384	97·0	2·18	1·92	2·47	0·98	372	97·1	2·19	1·94	2·46	0·92
Presence	12	3·0	2·19	1·80	2·65		11	2·9	2·20	1·83	2·66	

Table 2 *Continued*

	Total subjects						Subjects excluding those with high sFas level or a disease history					
	N	%	Adjusted means of sFas (ng mL^{-1})	95% CI		*P*-value*	*N*	%	Adjusted means of sFas (ng mL^{-1})	95% CI		*P*-value*
Number of metabolic risk factors												
			Trend $P = 0.97$						Trend $P = 0.75$			
0	54	13·6	2·21	1·91	2·56	Ref.	52	13·6	2·23	1·94	2·56	Ref.
1	143	36·1	2·17	1·90	2·47	0·63	140	36·6	2·15	1·90	2·44	0·39
2	131	33·1	2·19	1·92	2·49	0·79	126	32·9	2·19	1·94	2·48	0·71
3–4	68	17·2	2·19	1·91	2·51	0·86	65	17·0	2·22	1·95	2·53	0·98
Total	396	100·0					383	100·0				

Adjusted for age group, area, smoking status and drinking status.
Overweight/obesity; BMI ≥ 25 kg m^{-2}, Hypertension; ≥ 130/85 mmHg and/or medical history of hypertension, Hyperlipidaemia; serum triglycerides ≥ 1·69 mmol L^{-1} (150 mg dL^{-1}) or HDL-cholesterol < 1·03 mmol L^{-1} (40 mg dL^{-1}) for men and < 1·29 mmol L^{-1} (50 mg dL^{-1}) for women, Diabetes; medical history of diabetes.

Moreover, a clear association between sFas levels and the cluster number of metabolic risk factors was independently observed with age, smoking and drinking. In contrast, among women, we failed to find any clear association between the four metabolic risk factors and sFas levels.

Hebert *et al.* showed that plasma levels of sFas were associated with coronary artery disease in stable patients with end-stage renal disease [15]. Recently, a study examined plasma sFas levels in patients with coronary heart disease (CHD), CHD-equivalent, or 10-year CHD risk > 20% [16]. sFas levels were found to be statistically higher in such patients compared to healthy subjects as a result of our study. Moreover, they demonstrated that the sFas concentration was related to different cardiovascular risk factors such as diabetes, metabolic syndrome, and hypertension, and short-term treatment with atorvastatin, which has an anti-inflammatory effect, lowered sFas concentrations in these patients. These results suggest that sFas may play an important role in inflammation processes at atherosclerotic lesions. Another prospective study on dialysis patients also revealed that increased plasma sFas levels were significantly associated with future cardiovascular endpoints [9], suggesting that sFas is a novel predictor of active atherosclerotic disease.

Metabolic disorders, such as obesity, hypertension, hyperlipidaemia and diabetes are well-known risk factors for cardiovascular disease [17–19]. They increase production of ROS and initiate the process of atherosclerosis [5]. Endothelial injury or exposure to ROS induces apoptosis of endothelial cells, which leads to endothelial cell loss and results in atherogenesis and a procoagulative state [1]. The Fas–FasL system is one of the important death factors causing apoptosis to cells [20]. In contrast, sFas, a splicing variant of Fas, binds FasL and acts as a competitive antagonist of Fas apoptotic signalling [21]. Thus, sFas may interfere with the apoptotic pathway in the process of atherosclerosis and contribute to dysregulated inflammation [22].

Gender differences were observed in the association of metabolic risk factors and sFas levels; a positive association was found in men and no association in women. Most prior studies on atherosclerosis or metabolic disorders and sFas levels did not show results separated by gender [9,15,16]. Our cross-sectional study was not designed to determine the mechanisms of the gender differences. However, the result suggests that gender differences exist in the process of atherosclerosis through inflammatory response. For example, inflammation measured by C-reactive protein (CRP) levels showed a weaker effect on mortality from cardiovascular disease in women than in men [3]. Another study found that atherosclerosis, measured by common carotid artery intima-media thickness, was related to CRP in men, but not in women [23]. These gender differences deserve further attention.

Some limitations of our study must be discussed. First, the definitions we used to categorize metabolic risk factors were somewhat different from the diagnostic criteria adopted today [13,24]. This is because we did not obtain some of the necessary information at the baseline survey. However, except for diabetes, our definitions were the very same as those adopted in a recent Japanese study [19]. The BMI level of ≥ 25 kg m^{-2}, used in this paper instead of waist circumference, was reported to correspond well to the Asian criterion for high waist

circumference of ≥ 90 cm in men and ≥ 80 cm in women [25]. Diabetes was defined based on a self-report as we had no records on serum fasting glucose. Self-reported medical histories were found to be accurate among Japanese workers [26]. In the US, among residents ≥ 45 years of age, substantial agreement was found between self-reports and medical records (κ = 0·71–0·80) for diabetes [27]. Thus, the metabolic risk factors we adopted in this paper likely indicate overweight/obesity, hypertension, hyperlipidaemia, and diabetes, accurately, and we found a clear association of sFas level with clusters of these four metabolic risk factors among men. Second, since not all the cohort participants provided blood samples, there was the possibility of selection bias. Further, HDL-cholesterol and triglyceride were not commonly examined at the time of the baseline survey, and the number of subjects we analysed was relatively small compared to the original controls of the nested case–control study. However, donation depended solely on the subject's intention, and the control selection in the nested case–control study was based only on matching information. The examination of HDL-cholesterol and triglyceride depended on the health check-up system of each area. Thus, any bias due to blood donation or subject selection should not seriously affect our results. Third, serum samples were stored for approximately 10 years at −80 °C. The stability of sFas in these cohort samples could not be determined because their levels were not measured at baseline. However, Ito *et al.* compared newly collected sera and frozen specimens stored for 9 years gathered from a variety of different individuals, and found no statistically significant difference in the distributions of sFas levels [14], indicating that the serum sFas level remained stable after long-term storage at −80 °C.

In conclusion, serum sFas levels appear to be associated with overweight/obesity, hyperlipidaemia and cluster of metabolic risk factors among men, suggesting that serum sFas may elevate to down-regulate increased apoptosis in atherogenesis processes.

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ORIGINAL PAPER

Evaluation of Oncotype DX Recurrence Score as a prognostic factor in Japanese women with estrogen receptor-positive, node-negative primary Stage I or IIA breast cancer

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Abstract

Purpose We sought to evaluate the use of the **Oncotype** DX Breast Cancer Assay for identifying candidates for adjuvant therapy in patients with estrogen receptor (ER)-positive, node-negative primary Stage I or IIA breast cancer.

Methods A retrospective case–control study was conducted on 40 patients who underwent surgery between 2000 and 2008. Cases ($n = 10$) were patients who had metastases after surgery. Controls ($n = 30$) were patients who did not develop metastases and were individually matched to their case with respect to age. All patients were analyzed with regard to age, tumor size, histological grade, HER2 status, and the values of Recurrence Score (RS), ER score and PgR score generated by **Oncotype** DX. We also divided the patients into low, intermediate or high-risk groups according to individual RS values.

Results RS, risk category and histological grade were associated with metastases in patients with ER-positive, node-negative Stage I or IIA breast cancer. However, ER status, tumor size and PgR status were not associated with metastases. Histological grade was associated with RS value and the distribution pattern of risk category ($P < 0.001$ for each).

Conclusions Both histological grade and risk-category classification were effective in identifying women at risk of developing distant metastases after initial therapy for ER-positive, node-negative Stage I or IIA breast cancer. These patients may benefit from the addition of adjuvant therapy at diagnosis.

Keywords Oncotype DX · Japanese women · ER positive · Node negative · Adjuvant therapy · Breast cancer

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Introduction

More than 40,000 women are newly diagnosed with breast cancer each year in Japan, and the number of patients with breast cancer has been increasing. Breast cancer became the most common cancer in women in 1994, and it is the most common cause of death among women between the ages of 30 and 64 in Japan. About one-third of patients with breast cancer develop distant metastases after the initial surgical resection (Kamo et al. 2008; Matsuda et al. 2008), and this includes some women with estrogen receptor (ER)-positive, node-negative Stage I or IIA breast cancer. It is extremely

difficult to predict the risk of recurrence for this patient population, and the **Oncotype** DX system was developed to facilitate prognostic determination. **Oncotype** DX is a 21-gene assay that calculates the Recurrence Score (RS), a statistic designed to predict the risk of distant recurrence during a 10-year period, and predict the response to chemotherapy for lymph node-negative, hormone-positive breast cancer (Wolf et al. 2008). It uses reverse transcriptase-polymerase chain reaction (**Oncotype** DX Breast Cancer Assay, Genomic Health, Inc, Redwood City, CA, USA) to calculate an individualized RS using a proprietary algorithm. The RS has been prospectively validated as a predictor of 10-year recurrence-free survival in patients with node-negative, ER-positive early stage breast cancer (Paik et al. 2004).

However, most of the studies examining the **Oncotype** DX system have been conducted in the USA and Europe among Caucasian women, but the genetic background of patients with breast cancer can differ dramatically by race (Balan et al. 2008). This may have profound impacts on the clinical course and response to treatment. The purpose of this case–control study was to evaluate the utility of Oncotype DX in identifying Japanese women with ER-positive, node-negative Stage I or IIA primary invasive breast cancer who were candidates for adjuvant therapy.

Patients and methods

Patients

A case–control study was conducted under informed consent among 40 patients diagnosed with ER-positive, node-negative Stage I or IIA primary breast cancer who underwent surgery from January 2000 to December 2008 in Aichi Medical University and Marumo Hospital. Cases ($n = 10$) were consecutive patients who had distant breast cancer metastases after surgery. Controls ($n = 30$) were patients who did not have metastatic lesions during the observation period and who were individually matched to their case with respect to age (within 2 years). Patients underwent adjuvant hormone therapy or chemotherapy at the discretion of their individual doctors, and patients were excluded from the study if they had evidence of mucinous adenocarcinoma with a probability of a good prognosis.

Patient evaluation

Patients were analyzed with regard to such classical clinicopathologic features as age, tumor size, histological grade, HER2 status, lymphatic invasion and vascular invasion. All immunohistochemistry and histological determinations were performed by a single pathologist. We also analyzed the RS, the ER score and the PgR score generated by **Oncotype** DX Breast Cancer Assay for each patient. The ER score and the PgR score are included in the calculation of the RS. All of the patients were then classified into three groups based on the RS value as follows: low (RS < 18), intermediate (RS 18–30) or high (RS ≥ 31) risk-category groups. We compared the clinicopathologic characteristics of the case and control patients. The patients underwent the following evaluations every year: physical examination, blood tests, mammography, breast ultrasonography and computed tomography. The length of the follow-up period was calculated from the date of the first surgery to the date of the last visit to our outpatient clinic, and the presence of metastasis, death and cause of death were recorded for all appropriate patients.

Statistical analysis

All analyses were conducted using SPSS software, version 9.0. All confidence intervals (CI) are reported as 95% CI. Data are reported as the mean ± SD. When possible, all tests were two-sided, and $P < 0.05$ was considered significant for all analyses. Categorical variables were compared using the Fisher's exact test or Spearman's rank correlation coefficient. Continuous variables were compared using Student's t test. We conducted multivariable logistic regression analysis to determine whether RS or histological grade were associated with distant metastasis.

Oncotype DX

For eligible cases and controls, the **Oncotype** DX assay (Genomic Health Institute, Redwood City, CA, USA) was performed on preserved paraffin blocks according to the manufacturer's instructions.

Results

Table 1 lists the patient and tumor characteristics of the study population. All of the patients included in this study underwent surgery with an axillary dissection or a sentinel node biopsy. Among the cases ($n = 10$), two patients underwent breast conserving surgery, and eight patients underwent mastectomy. For the controls ($n = 30$), 19 patients had breast conserving surgery and 11 underwent mastectomy ($P = 0.028$). One of the case patients was given chemotherapy (CEF six courses) and four control patients had chemotherapy. These patients were all given different treatment regimens as follows: CMF three courses, EC four courses, CEF four courses and DOC four courses, and CMF six courses. There were no statistically significant differences between the cases and controls for the following parameters:

Table 1 Study population

	Cases	Controls	*P* value
No. of patients	10	30	
Age (year)	49.1 ± 12.9 (37–76)	50.9 ± 11.9 (37–78)	0.693
Tumor size (mm)	18.9 ± 4.6 (95% CI 15.7–22.2)	15.8 ± 6.3 (95% CI 13.4–18.2)	0.165
T1	6 (60%)	23 (77%)	0.418
T2	4 (40%)	7 (23%)	
Histological grade			
1	1 (10%)	15 (50%)	0.001
2	1 (10%)	9 (30%)	
3	8 (80%)	5 (17%)	
Unknown	0 (0%)	1 (3%)	
HER2 status			
Negative	7 (70%)	27 (90%)	0.057
Positive	2 (20%)	0 (0%)	
Unknown	1 (10%)	3 (10%)	
ly			
Positive	1	2	1.000
Negative	9	28	
v			
Positive	0	0	
Negative	10	30	
ER score	9.1 ± 1.7 (95% CI 7.9–10.3)	10.1 ± 1.3 (95% CI 9.6–10.5)	0.068
PgR score	6.6 ± 2.0 (95% CI 5.2–8.0)	7.7 ± 1.5 (95% CI 7.1–8.3)	0.069
RS	40.0 ± 26.4 (95% CI 21.1–58.9)	17.8 ± 10.9 (95% CI 13.8–21.9)	<0.001
Risk category group			
Low risk	3 (30%)	19 (63%)	0.005
Intermediate risk	1 (10%)	8 (27%)	
High risk	6 (60%)	3 (10%)	
Procedure performed			
Mastectomy	8 (80%)	11 (37%)	0.028
Partial mastectomy	2 (20%)	19 (63%)	
Adjuvant therapy			
Hormone therapy	9 (90%)	26 (87%)	0.999
Chemotherapy	1 (10%)	4 (13%)	

age at diagnosis, tumor size, HER2 status, lymphatic invasion, vascular invasion, the ER score, the PgR score and the type of the adjuvant therapy. The median follow-up period was 53.4 months for the cases and 55 months for the controls. When the cases were compared to the controls, the mean RS value of the cases [40.0 (95% CI 21.1–58.9)] was significantly ($P < 0.001$) higher than that of the controls [17.8 (95% CI 13.8–21.9)], and the proportion of patients in the RS risk categories also differed ($P = 0.005$) such that more cases were classified as high risk. Finally, there were significant differences between the case and control patients in the tumor histological grades ($P = 0.001$), and more cases had a histological grade 3 tumor than control patients.

We then examined the patients with a histological grade 3 tumor ($n = 13$) in greater detail and found a significant difference only in the distribution of the frequency of the type of adjuvant therapy between the patients with metastases ($n = 8$) and those without metastases ($n = 5$; $P = 0.035$; Table 2). However, when a similar analysis was performed with the patients stratified by RS-based high-risk category, a marginally significant difference was seen in treatment regimen ($P = 0.083$; Table 3).

Table 4 lists the characteristics of the patients treated with adjuvant hormone therapy. There were significant differences in RS ($P < 0.001$), risk category group ($P < 0.001$), histological grade ($P = 0.003$) and the PgR score ($P = 0.034$) between the patients with metastases ($n = 9$) and those without metastases ($n = 26$).

Among the patients with metastatic disease, four patients died as a result of progression of their metastatic breast

Table 2 Adjuvant therapy for patients with histological grade 3 tumor

	Cases	Controls	*P* value
No. of patients	8	5	
Adjuvant therapy			
Hormone therapy	8	2	0.035
Chemotherapy	0	3	

Table 3 Adjuvant therapy for patients classified into high risk group

	Cases	Controls	*P* value
No. of patients	6	3	
Adjuvant therapy			
Hormone therapy	6	1	0.083
Chemotherapy	0	2	

cancer, and the first site of metastasis for each patient is shown in Table 5. The median disease free survival period for these patients was 17 months. Among the four patients who died of metastatic disease, the median interval to recurrence was 18 months, and the median time from surgery to death was 56 months.

We also examined the distribution of the RS value and the distribution of RS category according to histological grade for all of the patients. Histological grade was strongly associated with the RS value ($P < 0.001$; Fig. 1) and the distribution of RS category ($P < 0.001$, $\rho = 0.63$; Table 6).

Multivariable logistic regression analysis was performed to explore the relation between distant metastasis and age, ER score, PgR score, RS, histological grade and lymphatic invasion. Histological grade 3 was significantly associated with distant metastasis (Table 7).

Discussion

The prognosis for the patients with ER-positive, node-negative Stage I or IIA breast cancer is better than that for patients with more advanced invasive breast cancer, but, even within this low-stage group, some patients experience disease recurrence or metastasis and ultimately die of metastatic disease. It remains difficult to reliably predict the prognosis of patients with ER-positive, node-negative Stage I or IIA breast cancer, but the Onco*type* DX system was developed to assist in prognostic determination of this patient group. Some studies have estimated the risk category for patients, and this has been incorporated into treatment decisions (i.e., the need for adjuvant therapy) for patients with invasive breast cancer in the United States. However, no one has examined the utility of using RS in the treatment algorithm of women with invasive breast cancer in Japan. The RS incorporates several different biological parameters into its calculation, and it is possible that RS may not have the same degree of accuracy in patients with different genetic background.

We stratified the patients into three risk categories based on their RS, and found a significant difference in histological grades for each risk category between patients with and without metastases. Furthermore, patients with both metastases and a tumor of histological grade 3 ($n = 8$), were significantly more likely to have received hormone therapy than patients with a histological grade 3 tumor who remained cancer free ($n = 5$; $P = 0.035$). However, when patients with ($n = 6$) or without ($n = 3$) metastases were classified into the high-risk category based on their RS, there was a marginally significant difference in the type of adjuvant therapy used ($n = 3$; $P = 0.083$). Our sample size

Table 4 Patient characteristics treated with hormone therapy

	Cases	Controls	*P* value
No. of patients	9	26	
Age (year)	50.0 ± 13.4 (37–76)	52.3 ± 11.9 (37–78)	0.636
Tumor size	18.4 ± 4.5 (95% CI 14.9–21.8)	15.8 ± 6.4 (95% CI 13.2–18.5)	0.28
Histological grade			
1	1 (10%)	15 (58%)	0.003
2	1 (10%)	8 (31%)	
3	8 (80%)	2 (8%)	
Unknown	0 (0%)	1 (4%)	
RS	42.9 ± 26.3 (95% CI 22.7–63.1)	16.2 ± 10.3 (95% CI 12.0–20.4)	<0.001
Risk category group			
Low risk	2 (22%)	18 (69%)	<0.001
Intermediate risk	1 (11%)	7 (27%)	
High risk	6 (67%)	1 (4%)	
ER score	9.1 ± 1.8 (95% CI 7.7–10.5)	10.2 ± 1.4 (95% CI 9.6–10.7)	0.073
PgR score	6.5 ± 2.1 (95% CI 4.9–8.1)	7.8 ± 1.4 (95% CI 7.3–8.4)	0.034

Table 5 Case characteristics

Characteristic	Patient no.									
	1	2	3	4	5	6	7	8	9	10
Age at diagnosis (year)	41	37	64	76	55	41	37	53	47	40
Size (mm)	24	18.2	18	27	16	15	21	21	18	11
Histological grade	2	3	3	1	3	3	3	3	3	3
HER2	1+	1+	1+	–	3+	–	3+	1+	–	Unknown
ER score	8.88	10.1	9.71	11.81	9.33	8.03	6.24	7.11	8.8	10.84
PgR score	7.73	8.2	5.42	9.46	2.99	6.51	5.09	4.89	7.27	8.42
Recurrence Score	14	37	32	12	75	20	72	74	52	12
Risk category group	1	3	3	1	3	2	3	3	3	1
Adjuvant therapy	C	H	H	H	H	H	H	H	H	H
Interval from surgery to recurrence (month)	37	12	29	5	62	54	18	18	52	43
The first site of metastasis	Bone	Brain	Lung	Bone	Brain	Bone	Lung	LN	Lung	Lung
Outcome	Alive	Alive	Alive	Dead	Alive	Alive	Dead	Dead	Alive	Dead
Interval from surgery to death (month)				108			48	27		64

C chemotherapy, *H* hormone therapy

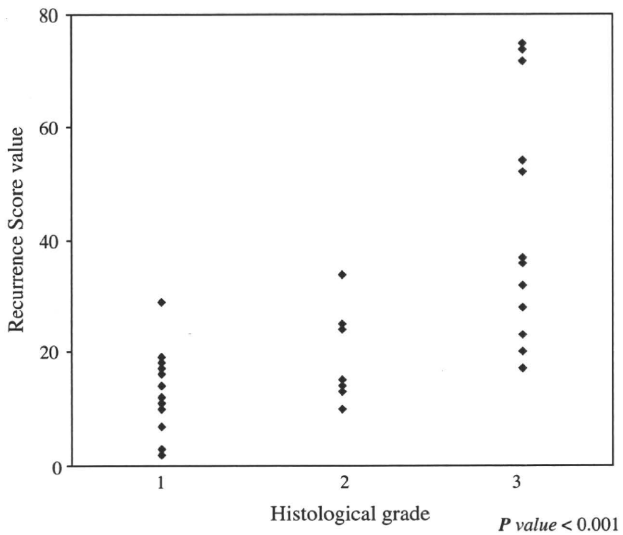

Fig. 1 The distribution of the RS value according to the histological grade. $P < 0.001$

was likely too small, though, to detect any significant differences for this parameter.

We found significant differences in the proportion of the tumor histological grade ($P = 0.001$) and the RS risk category ($P = 0.005$) and the mean RS value ($P < 0.001$) between the case and control patients. This data suggest the tumor histological grade is stronger prognostic power than the RS risk category. However, biopsies and even surgical specimens are subject to sampling and pathologist error, and there can be also interobserver disagreement in nuclear atypia scoring of node-negative breast cancers (Tsuda et al. 1999). By relying upon biochemical and genetic markers of disease, RS generated by **Onco*type*** DX is objectively

Table 6 The distribution of the risk category group according to the histological grade

Risk category group	Histological grade		
	1	2	3
Low risk	13	7	2
Intermediate risk	3	2	3
High risk	0	1	8

$P < 0.001$, $\rho = 0.63$

Table 7 Multivariate logistic regression analysis of age, ER score, PgR score, RS, histological grade, ly in relation to the likelihood of distant metastasis

Variable	*P* value	Odds ratio (95% CI)
Age at diagnosis	0.195	0.90 (0.764–1.057)
ER score	0.651	1.33 (0.389–4.53)
PgR score	0.378	0.65 (0.246–1.702)
RS 50 or more vs RS less than 50	0.579	2.85 (0.07–115.552)
Histological grade 2 vs histological grade 1	0.369	7.48 (0.093–602.504)
Histological grade 3 vs histological grade 1	0.041	222.0 (1.243–39647.336)
ly(+) vs ly(−)	0.557	0.37 (0.013–10.312)

determined and independent of the skill and diagnostic acumen of pathologists. Additionally, these biomarkers may better reflect cancer pathophysiology than morphology alone. Our data also suggest that the RS value has a stronger prognostic power than the tumor histological grade. It seems to be valid to regard the RS value, with a universal