Micronuclei levels in peripheral blood lymphocytes as a potential biomarker for pancreatic cancer risk

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To find biomarkers for risk prediction of pancreatic cancer (PC), we evaluated the frequency of micronuclei (MN) in peripheral lymphocytes of 346 patients with PC and 449 healthy controls. The levels of baseline MN (mean ± standard error of micronucleated cells per 1000 binucleated cells) were significantly higher in patients (15.3 ± 0.3) than those in controls [9.7 ± 0.5; adjusted for body mass index (BMI), $P < 0.001$]. Using the median levels found in controls as the cut point, 78.9% of patients and 43.7% of controls had a higher frequency of MN. Logistic regression analysis with adjustment for known risk factors for PC showed that having a higher level of MN was significantly associated with increased risk of PC [odds ratio (OR): 8.32, 95% confidence interval (CI): 5.06–13.67, $P < 0.001$]; and the risk was much higher in men than in women [OR (95% CI): 14.19 (7.09–28.40) versus 4.19 (1.90–9.27)]. The level of MN was not associated with disease stage or resection status but was related to smoking status in men and to BMI in women among patients. The level of MN was higher in smokers (14.5 ± 0.6) than in nonsmokers (12.1 ± 0.6; $P = 0.023$) and in obese (25.3 ± 2.8) versus normal weight individuals (17.7 ± 0.8; $P = 0.024$). These data showed that elevated level of MN in peripheral lymphocytes was associated with increased risk of PC.

Introduction

Pancreatic cancer (PC) is the fourth leading cause of cancer death in the USA. It is a rapidly fatal disease with a 5 years survival rate of <5% (1). Known risk factors for PC include cigarette smoking, obesity and type II diabetes, a family history of PC, and several inherited familial syndromes. Some of these factors may share a common mechanism involving oxidative stress and inflammation (2,3). Novel approaches are needed to identify and validate biomarkers for risk prediction and early detection of PC.

PC shows extensive genomic instability and aneuploidy. Telomere attrition and mutation in *TP53* and *BRCA2* genes, as well as defects in the mitotic spindle apparatus conferred by centrosome abnormalities, may contribute to the aneuploidy and genomic instability seen in this type cancer (4). Centrosome abnormalities are detected in 85% of PC, and there is a correlation between levels of such abnormalities and the degree of chromosomal aberrations present (5). It is well known that chromosomal aberrations, such as the background micronuclei (MN), reflect accumulative DNA damage as a result of exposure to genotoxic agents and individual variations in response to DNA damage (6). On the other hand, the level of chromosomal/DNA damage in cultured peripheral lymphocytes that are *in vitro* treated with genotoxic agents, such as tobacco carcinogens, mutagens and H_2O_2 under the same exposure conditions, has been used extensively in molecular epidemiology as a phenotypic marker for individual variations in DNA repair (7).

Abbreviations: BMI, body mass index; CI, confidence interval; CV, coefficient of variation; HUMN, Human MicroNucleus; MN, micronuclei; OR, odds ratio; PC, pancreatic cancer.

MN originate from chromosomal fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division (8). Compared with other cytogenetic assays, the MN assay is highly reliable, easier to score and less costly. The Human MicroNucleus (HUMN) project involving >30 laboratories worldwide has provided technical guidelines, identified sources of variations and conducted prospective validation studies on this biomarker (9–11).

MN levels have been extensively used as biomarkers of genetic damage in humans with known exposure to genotoxic agents. The value of MN as a cancer susceptibility marker has been supported by numerous observations. For example, a number of studies have found elevated levels of MN formation in the peripheral lymphocytes of cancer patients prior to chemotherapy or radiotherapy and in patients affected by cancer-prone congenital diseases such as Bloom syndrome or ataxia telangiectasia (9,12,13). Levels of MN in the oral mucosa have been used as surrogate end points in clinical chemoprevention trials for oral malignancies (14). Many MN-inducing genotoxic agents are known to be carcinogenic and the HUMN project has confirmed that an elevated MN frequency in peripheral blood lymphocytes predicts an increased cancer risk (15). A nested case–control study based on a longitudinal cohort study also provided supporting evidence for the efficacy of MN levels as a biomarker for cancer risk in the general population (16).

To demonstrate the associations between the risk of PC and the level of intrinsic chromosome instability and sensitivity to oxidative stress-induced chromosomal damage, we conducted the cytokinesis-block MN assay (8,17) in a large group of patients with pancreatic adenocarcinoma and healthy controls. To our knowledge, this is the largest group of patients in whom this has been studied. We report that the background and hydrogen peroxide (H_2O_2)-induced MN levels in peripheral lymphocytes are predictive of the risk of PC.

Materials and methods

Study population

Our study design and data collection methods have been described previously in detail (18). Briefly, in this hospital-based case–control study, 346 patients with newly diagnosed and pathologically confirmed primary pancreatic ductal adenocarcinoma and 449 healthy controls were consecutively recruited from The University of Texas M. D. Anderson Cancer Center between January 2002 and June 2006. The response rate (number of individuals enrolled per number of individuals approached) was 83.9% for cases and 83.6% for controls (19). Control individuals were cancer-free and selected from friends and genetically unrelated family members (usually spouses or in-laws) of patients who were diagnosed with cancers other than gastrointestinal cancer, lung cancer or head and neck cancer. Patients and control individuals were frequency matched by age (±5 years), gender and race. In personal interviews, we collected information on cigarette smoking, alcohol consumption, diabetic status, medical history, family history of cancer among first-degree relatives and body mass indices (BMIs) at different age period (19). Because we did not include BMI in the questionnaire until 2004, this information was missing for 106 patients and 287 controls. We created a variable for missing BMI information (yes or no) and this variable was adjusted for in all data analysis. A blood sample was collected from each study participant in heparinized vacutainers. The median time interval between the initial diagnosis and blood drawn was 7 days [95% confidence interval (CI): 1–9]. Patients with PC who had received cytotoxic chemotherapy before the blood collection were excluded from the current study. For cancer patients, we also conducted medical record review to collect information on disease stage, tumor resection status and overall survival time. Each participant gave written informed consent in a protocol approved by the M. D. Anderson Institutional Review Board.

Smokers were defined as individuals who had smoked ≥100 cigarettes during their lifetimes. Cumulative smoking was measured by pack-years smoked, with heavy smokers defined as those having smoked >20 pack-years. Ever-alcohol drinkers were defined as individuals who had consumed at least four alcoholic drinks of beer, wine or hard liquor each month for 6 months in

MN frequency and risk of PC

The background and H_2O_2-induced (mean ± standard error) were significantly higher in patients (15.3 ± 0.3 and 19.2 ± 0.4) than in controls (10.7 ± 0.3 and 14.5 ± 0.4) ($Ps < 0.001$). The level of H_2O_2-induced MN paralleled the level of background MN (correlation coefficient = 0.846, $P < 0.001$). Because of the well-known sex difference in MN level, we selected the median value of the controls in each sex group (12/1000 cells in women and 9/1000 cells in men) as the cut point for MN high and low categorization (Table II). In total, 78.9% of the patients and 43.7% of the controls had a higher than median level of background MN. Logistic regression analysis with adjustment for demographic factors and known risk factors (including BMI) for PC found that having a higher frequency of background MN was significantly associated with an increased risk of PC [OR and 95% CI: 8.32 (5.06–13.67), $P < 0.001$]. The MN-associated risk of PC was much higher in men than in women [OR (95% CI): 14.19 (7.09–28.40) versus 4.19 (1.90–9.27)] (Table II). A higher level of H_2O_2-induced MN (>14/1000 cells for both men and women) was also significantly associated with increased risk of PC [OR (95% CI): 3.38 (2.05–5.57), $P < 0.001$].

To determine if the levels of MN in the lymphocytes could significantly differentiate between PC patients and healthy controls, receiver operating characteristic curves were constructed. The area under the curve (95% CI) was 0.73 (0.69–0.76, $P < 0.001$) for the background MN and 0.68 (0.64–0.72, $P < 0.001$) for the H_2O_2-induced MN (Figure 1). Using the optimal cut point of 11.5 for background MN and 15.5 for H_2O_2-induced MN, the sensitivity and specificity was 70.2 and 63.5% and 62.1 and 61.8%, respectively.

Factors influencing the level of MN

Because of the significant differences in the distribution of the MN frequencies between patients and controls, we analyzed the impact of age, race, sex, smoking, alcohol, diabetes, BMI and family history of cancer on MN frequency by case–control strata (Table III) A significantly higher frequency of both baseline and H_2O_2-induced MN level was detected in women than in men for both patients and control groups. We also found that the frequency of background MN in patients with PC was higher in those of >60 years of age (15.9 ± 0.5, n = 201) compared with those of ≤60 years of age (14.3 ± 0.4, n = 145; $P = 0.049$, Table III). The difference became nonsignificant after adjusting for BMI ($P = 0.059$). Among controls, MN was negatively associated with BMI (Person's correlation coefficient = −0.190 and $P = 0.015$). We also noticed a significantly higher level of MN in controls without the BMI information

than those with the BMI information and this difference could not be explained by the age, sex and smoking distribution between the two groups (data not shown). Thus, we created a variable of BMI information (yes or no) and included this variable in the data analysis.

Because of the significant sex difference in MN level, we further analyzed the data by sex strata. Among female patients, those who were obese had a higher frequency of background MN (25.3 ± 2.8, n = 6) compared with normal weight patients (17.7 ± 0.8, n = 73; $P = 0.024$). Among men, cigarette smoking had a differential effect on MN formation for both patients and controls. In male patients, a higher frequency of background MN was observed in ever smokers (14.5 ± 0.6, n = 92) than in nonsmokers (12.1 ± 0.6, n = 49) ($P = 0.023$). In contrast, among men serving as controls, a lower frequency of background MN was detected in heavy smokers (8.3 ± 0.6, n = 82) than in nonsmokers (9.0 ± 0.4, n = 89; $P = 0.014$). Because BMI is a major risk factor for PC, we also examined the impact of smoking on MN with adjustment for BMI among men (Table IV). We found a significantly higher frequency of baseline MN in ever smokers than nonsmokers among patients but not among controls. We did not observe any substantial differences in MN frequencies by other factors examined.

Discussion

Our observations add to existing evidence supporting the value of MN as a cancer susceptibility biomarker (13). In the present hospital-based case–control study, a significant increase in baseline and H_2O_2-induced MN frequency in peripheral lymphocytes was observed in patients with PC over healthy controls. This higher level of MN was associated with an 8-fold increase in risk of PC. Furthermore, we have for the first time demonstrated a differential association of smoking and levels of background MN between male patients and controls and a lack of association between levels of background MN and disease stage.

MN has been successfully used as a marker for DNA damage in populations with known exposure to genotoxic agents. Our study has demonstrated an association between levels of MN formation and the risk of PC. It is possible that the higher frequency of MN in patients with PC reflects the accumulated genetic alterations caused by known and suspected genotoxic factors, such as cigarette smoking, obesity and heavy alcohol consumption as well as individual variations in susceptibility to these factors. However, it is difficult to establish

Table II. Background MN frequency and risk of PC

MN	Patient		Control		OR (95% CI)[a]	OR (95% CI)[b]
	N	%	N	%		
Overall[c]						
Low	73	21.1	253	56.3	1.0	1.0
High	273	78.9	196	45.7	4.75 (3.40–6.63)	8.32 (5.06–13.67)
≤10	74	21.4	247	55.0	1.0	1.0
>10	272	78.6	202	45.0	5.96 (4.14–8.57)	9.0 (5.16–15.68)
Women						
≤12	42	28.2	113	53.3	1.0	1.0
>12	107	71.8	99	46.7	2.92 (1.80–4.74)	4.19 (1.90–9.27)
Men						
≤9	31	15.7	140	59.1	1.0	1.0
>9	166	84.3	97	40.9	7.25 (4.50–11.69)	14.19 (7.09–28.40)

[a]OR was adjusted for age, sex, race, smoking, alcohol consumption, history of diabetes and family history of cancer.
[b]OR was adjusted for age, sex, race, smoking, alcohol consumption, history of diabetes, family history of cancer and BMI.
[c]Overall: low and high used the median control value in women (≤12 or >12) and men (≤9 or >9) separately. The median control value of the control group including both women and men was 10.

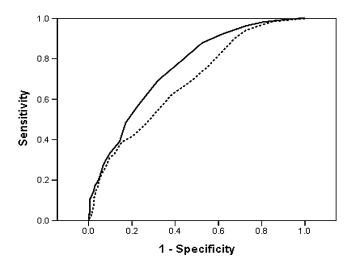**Fig. 1** Receiver operating characteristic curve for MN and PC case–control status. The area under the curve (95% CI) was 0.73 (0.69–0.76, $P < 0.001$) for the background MN (solid line) and 0.68 (0.64–0.72, $P < 0.001$) for the H_2O_2-induced MN (dashed line). Using the optimal cut-point of 11.5 for background MN and 15.5 for H_2O_2-induced MN, the sensitivity and specificity was 70.2 and 63.5% and 62.1 and 61.8%, respectively.

Table III. Factors influencing the level of MN in patients and controls

Variables	Background						H$_2$O$_2$ induced					
	Patients			Controls			Patients			Controls		
	n	Mean ± SE	P[a]	n	Mean ± SE	P[a]	n	Mean ± SE	P[a]	n	Mean ± SE	P[a]
Overall	346	15.3 ± 0.3		449	10.7 ± 0.3		322	19.2 ± 0.4		322	14.5 ± 0.4	
Age												
≤60	145	14.3 ± 0.4	Ref.	224	10.7 ± 0.3	Ref.	134	18.6 ± 0.6	Ref.	157	14.9 ± 0.5	Ref.
>60	201	15.9 ± 0.5	0.049	225	10.7 ± 0.4	0.999	188	19.7 ± 0.6	0.982	165	14.0 ± 0.6	0.152
Sex												
Women	149	17.1 ± 0.6	Ref.	212	13.0 ± 0.5	Ref.	136	22.0 ± 0.8	Ref.	147	17.7 ± 0.7	Ref.
Men	197	13.4 ± 0.3	<0.001	137	8.9 ± 0.3	<0.001	186	17.0 ± 0.4	<0.001	175	12.1 ± 0.4	<0.001
Race/ethnicity												
White	293	15.3 ± 0.3	Ref.	409	10.7 ± 0.3	Ref.	271	19.2 ± 0.4	Ref.	296	14.5 ± 0.4	Ref.
Non-white	28	15.5 ± 0.9	0.418	22	10.8 ± 1.1	0.539	28	19.6 ± 1.0	0.278	14	14.3 ± 1.4	0.629
Smoking												
Never-smoker	132	15.0 ± 0.4	Ref.	226	11.6 ± 0.3	Ref.	121	19.2 ± 0.6	Ref.	169	15.3 ± 0.5	Ref.
Former smoker	160	15.3 ± 0.5	0.303	169	9.7 ± 0.5	0.077	150	19.1 ± 0.7	0.225	114	13.4 ± 0.8	0.505
Current smoker	54	15.8 ± 0.7	0.256	54	9.9 ± 0.7	0.216	51	20.0 ± 1.0	0.266	39	14.3 ± 1.1	0.951
≤20 pack-years	87	15.5 ± 0.5	0.171	116	10.3 ± 0.4	0.319	83	18.7 ± 0.7	0.379	76	14.3 ± 0.7	0.809
>20 pack-years	127	15.3 ± 0.7	0.402	107	9.4 ± 0.7	0.023	118	19.7 ± 0.8	0.159	77	13.0 ± 1.0	0.560
Alcohol												
Never	151	15.6 ± 0.5	Ref.	207	11.8 ± 0.5	Ref.	138	20.1 ± 0.7	Ref.	154	15.8 ± 0.6	Ref.
<60 g/day	154	15.3 ± 0.4	0.422	203	10.0 ± 0.4	0.447	148	18.5 ± 0.6	0.702	141	13.8 ± 0.6	0.554
≥60 g/day	41	13.2 ± 0.6	0.928	35	9.6 ± 0.7	0.969	36	17.7 ± 0.9	0.862	24	11.5 ± 1.1	0.257
Diabetes												
No	254	15.5 ± 0.4	Ref.	406	10.7 ± 0.3	Ref.	237	19.6 ± 0.5	Ref.	289	14.5 ± 0.4	Ref.
Yes	92	14.2 ± 0.6	0.186	43	11.5 ± 0.9	0.333	85	17.9 ± 0.7	0.167	33	15.3 ± 1.2	0.243
Family history of cancer												
No	117	14.9 ± 0.5	Ref.	202	10.2 ± 0.3	Ref.	107	18.9 ± 0.6	Ref.	156	14.0 ± 0.5	Ref.
Yes	229	15.5 ± 0.4	0.536	247	11.0 ± 0.4	0.101	215	19.4 ± 0.5	0.574	166	14.8 ± 0.6	0.428
BMI (kg/m^2)												
<25	128	16.3 ± 0.5	Ref.	95	10.6 ± 0.6	Ref.	127	19.8 ± 0.7	Ref.	95	14.5 ± 0.8	Ref.
25–30	80	13.5 ± 0.5	0.197	56	8.3 ± 0.6	0.412	79	16.3 ± 0.6	0.083	54	11.3 ± 0.8	0.648
>30	32	16.6 ± 1.3	0.178	11	7.4 ± 2.3	0.226	32	20.7 ± 1.7	0.193	11	10.4 ± 3.0	0.292
Disease stage												
Localized	32	14.4 ± 1.3	Ref.	NA			30	18.8 ± 1.5	Ref.	NA		
Locally Advanced	89	15.6 ± 0.8	0.375				80	19.7 ± 0.9	0.608			
Metastatic	155	14.7 ± 0.6	0.933				146	18.3 ± 0.7	0.620			
Unstaged	51	16.7 ± 1.0	0.433				49	21.0 ± 1.2	0.358			

[a]Analysis of covariance test for differences in MN frequency between subgroups with adjustment for age, race/ethnicity, smoking, alcohol consumption, diabetes and family history of cancer.

Table IV. Background MN frequency between nonsmokers and smokers in men

Variables	Patients			Controls		
	n	Mean ± SE	P[a]	n	Mean ± SE	P[a]
Without BMI adjustment						
Nonsmoker	49	12.12 ± 0.56	Reference	36	8.14 ± 0.66	Reference
Smoker	92	14.48 ± 0.62	0.023	59	7.25 ± 0.79	0.593
≤20 pack-years	33	13.87 ± 0.81	0.106	27	7.61 ± 0.90	0.909
>20 pack-years	59	14.59 ± 0.88	0.027	32	7.38 ± 1.23	0.323
With BMI adjustment[b]						
Nonsmoker	49	12.12 ± 0.56	Reference	36	8.14 ± 0.66	Reference
Smoker	92	14.50 ± 0.62	0.023	59	7.22 ± 0.79	0.593
≤20 pack-years	33	13.79 ± 0.82	0.104	27	7.71 ± 0.91	0.913
>20 pack-years	59	14.65 ± 0.88	0.027	32	7.27 ± 1.24	0.326

[a]Analysis of covariance test for differences in MN frequency between subgroups with adjustment for age, race/ethnicity, alcohol consumption, diabetes and family history of cancer.
[b]BMI was adjusted as a continuous variable.

a causal relationship between increased MN levels and cancer risk because a high MN level in cancer patients could be a consequence of the disease (known as reverse causality) or reflect an individual's susceptibility to events causing genomic instability (9). The predictive value of background levels of MN as a biomarker for cancer risk in the general population has been preliminarily validated by two ongoing cohort studies, which involve plans to extend the length of follow-up for those study cohorts (15,16). The lack of association between the level of MN and disease stage argue against the possibility that the observed higher level of MN was mainly due to the disease.

There is a growing awareness that oxidative stress and/or inflammation plays a contributory role in numerous pathologies, including pancreatic carcinogenesis (3). To find out whether individual susceptibility to oxidative stress-induced DNA damage was associated with risk of PC, we measured the MN frequency in peripheral lymphocytes treated *in vitro* with H$_2$O$_2$. We found that the background and H$_2$O$_2$-induced MN frequencies were parallel, i.e. those with a higher level of background MN also had a higher level of H$_2$O$_2$-induced MN, suggesting that the individual did indeed have an increased susceptibility to oxidative stress. These observations suggest an association between elevated level of background MN and increased risk of PC.

The current study confirmed that MN formation differs according to age and sex as these were the demographic variables that most consistently predicted the levels of MN found in peripheral lymphocytes (21–25). The effect of age seems to reflect the accumulated genetic damage over a lifetime. The sex difference could be ascribed to an aneuploidy phenomenon, i.e. the X chromosome tends to lag behind in female lymphocyte anaphase, being micronucleated more efficiently than autosomes (26).

Previously, reports on the association of smoking with background MN frequency are inconsistent. Despite a few reports showing a positive association (27–29), the majority of studies did not find any association between levels of MN frequency and smoking (15,16,30–32). In men, we observed different associations between smoking and MN levels between patients and controls. Among patients, smokers, especially heavy smokers, had higher levels of MN than nonsmokers; however, among men in the control group, heavy smokers had a lower frequency of MN than light or never-smokers. Our observation in the control group was in agreement with the results reported by the HUMN project that current and former smokers experienced a small decrease of MN frequencies compared with never-smokers (32). These observations suggest that the higher level of MN in patients who had smoked is largely determined by genetic susceptibility to such exposure because smokers in the control group did not have increased level of MN. The lower levels of MN among men in the control group who had smoked heavily may have induced some protective mechanisms, e.g. detoxification enzymes and DNA repair enzymes that actually led to a lower level of MN. In addition, smoking may induce apoptosis and the micronucleated cells may be preferentially eliminated by apoptosis or the damaged cells may not divide and fail to form binucleated cells (32). On the other hand, patients may have more cells with DNA damage because of their deficiency in apoptosis signaling. It is not known whether female hormones have a protective effect against DNA damage that could have negated the effects of smoking among women, but the question needs further investigation.

Interestingly, we observed a significant negative association between BMI and MN level in controls but a positive association between obesity, an established risk factor for PC, and MN levels among female patients (19). Increased oxidative stress and inflammatory conditions could be underlying mechanisms contributing to the elevated levels of MN in obese women; but the negative association between BMI and MN in controls did not support this hypothesis. Whether there is a difference in host response to oxidative DNA damage between patients and controls that lead to the differential effect of BMI on MN needs further investigation. The small sample size for the obese group also suggests these observations need to be confirmed in a larger study.

The strengths of the current study include a large overall sample size, the collection of detailed epidemiologic information and having a single researcher scoring all the samples to control for observer variations. The limitations of the study include the case–control design and its inherent recall bias. However, the risk estimates for major risk factors such as smoking, obesity, diabetes and family history of PC in our study were quite comparable with that reported in cohort studies (18,19), which do not suggest a significant recall bias. Other limitations include the unbalanced match on race/ethnicity, incompleteness of the data on BMI and the small sample size in some of the subgroup analyses. Additionally, only actual chromosome breakage (in the form of micronucleated cells) was measured in our study, so other chromosomal damage endpoints, such as chromosome rearrangements as indicated by nucleoplasmic bridges and gene amplification as indicated by nuclear buds, were not evaluated. The impact of dietary nutrients (33) and genetic variations in DNA damage response also need further study.

In summary, data from this study provide convincing evidence that elevated levels of MN in peripheral lymphocytes are associated with risk of PC. Other large-scale epidemiological studies in different populations, preferably in a prospective design, are warranted to confirm the causal role of MN formation in the occurrence of genetic instability and PC.

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