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Polymorphism -23*HPh*I in the promoter of insulin gene and pancreatic cancer: A pilot study

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Pancreatic cancer (PC) is one of the most frequent gastrointestinal malignancies with extremely poor prognosis. In spite of a relative low incidence of PC, in comparison with other cancers, PC is the fourth leading cause of cancer death in USA in both sexes. The available data clearly suggest that diabetes mellitus (DM) can be both a long-standing cause of PC and an early manifestation of the disease. Besides of DM, insulin resistance and high insulin levels are linked as well with increased cancer risk, including PC.

The variable number of tandem repeats (VNTR) locus upstream of the insulin gene (*INS*) regulates insulin expression and has been associated with susceptibility to many diseases including DM. It is known that there is nearly complete linkage disequilibrium of the insulin variable tandem of repeats (*INS*-VNTR) alleles I/III with neighboring -23 *Hph*I A/T single nucleotide polymorphism (SNP) in Caucasians. Therefore, we have studied the association between SNP of -23*Hph*I in promoter of *INS* with PC, DM Type 2 (2TDM) and healthy controls. In this study we investigated 153 subjects (86 M/67 F); 51 patients with newly-diagnosed PC (31 M/20 F), 45 patients with 2TDM (29 M/16 F) and 57 healthy control subjects (26 M/31 F). The polymorphism of -23*Hph*I (A/T) in the promoter of *INS* was determined by the combination of polymerase chain reaction (PCR) with the restriction fragment length polymorphism (RFLP) methods. The results obtained by the PCR-RFLP analyses of SNP -23*Hph*I were confirmed by a direct studied locus sequencing of the genomic DNA. The frequency of abnormal glucose metabolism (both DM and impaired fasting glucose) was 88 % (45/51) in PC group. The AA genotype in SNP -23*Hph*I was more prevalent (67 % vs. 47 %; P<0.05) among PC patients compared to controls. Additionally, statistically significant differences were found in frequencies (%) of genotypes AA/AT/TT in groups with PC (67/27/6), 2TDM (53/40/7) compared to healthy controls (37/46/17) (P<0.05). Moreover, a statistically significant effect of -23*Hph*I A/T polymorphism on tumor staging was found (P< 0.05). Polymorphism of -23*Hph*I (A/T) in the promoter of *INS* may play a role in the pathogenesis of PC and could contribute to tumor staging.

Key words: pancreatic cancer; insulin gene regulation; polymorphism of -23HphI; diabetes mellitus; disorders of glucoregulation

Pancreatic cancer (PC), one of the most frequent gastrointestinal malignancies, is a disease with extremely poor prognosis. The 5-year survival ranges from 0.4 to 2.0 %. In the USA, PC is the fourth leading cause of cancer –related death in both sexes (following cancers of lung, colorectum and prostate in men, and cancers of lung breast, and colorectum in women) [1]. In the Czech Republic, PC is the fourth most frequent malignancy in men and the third in women with around 1 700 new cases (for both sexes) estimated in 2005. A reported incidence of PC in Czech Republic (in 2005) was 18.6 (16.7 respectively) new cases per 100 000 in men (women respectively) [2].

The etiology and pathogenesis of PC remains unknown, but it is supposed to be multifactorial. Besides of demographic factors (old age, black race) and genetic risk factors [hereditary pancreatitis, familial pancreatic cancer, von Hippel-Lindau syndrome, *BRAC2*, Li-Fraumeni syndrome, etc.] several lifestyle and environmental factors, such as smoking, obesity and low physical activity have been re-

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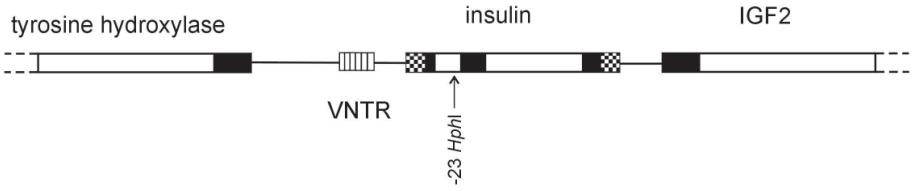

Figure 1. The insulin gene region on chromosome 11p15.5
Open, closed and checkered boxes stand for introns, exons, and untranslated regions, respectively. Relative position is indicated as the difference from the first base of the initiating codon AGT (= +1)
VNTR – the variable number of tandem repeat locus, IGF2 – insulin like growth factor 2.

ported to contribute to the development of the disease. Apart from other predisposing medical conditions (chronic pancreatitis, gastrectomy, cholecystectomy, *Helicobacter pylori* infection), obesity, insulin resistance and diabetes mellitus seem to be most important [3, 4, 5, 6, 7, 8].

The data available today suggest that diabetes mellitus (DM) can be both a long-standing cause of PC, and on the other hand, PC, as shown still more frequently *up now*, can be an early manifestation of DM [9, 10, 11]. The complex relationship between DM and PC has long been recognized. Concomitant presence of PC and DM is a well-known phenomenon and is described nearly in 30 % of PC patients, in comparison with the general population, in which the occurrence of DM is found in 6 – 7 % [12]. Prevalence of DM in PC patients varies from 4 do 64 % in published literature; more than 80 % of PC patients have DM or another disorder of glucose homeostasis (impaired fasting glucose, impaired glucose tolerance, respectively) at the time of PC diagnosis [13]. A recent meta-analysis of epidemiological studies of the association between DM and PC reported a combined age- and sex-adjusted odds ratio (OR) of 1.82 for PC among patients with DM, whereas the duration of DM revealed also an impact on the risk of malignancy [9]. On the other hand, there is a growing evidence that PC can cause DM [11, 14].

Based on the findings from several retrospective and prospective observational studies, 2TDM, impaired glucose tolerance, insulin resistance and hyperinsulinaemia are risk factors of PC [7, 9, 15]. One of biologically plausible mechanisms possibly relating the states of insulin resistance to pancreatic carcinogenesis is through growth-regulatory effects of the insulin-like growth factor system, which consists of insulin and insulin-like growth factors (IGF-1, IGF-2), their receptors and insulin-like growth factor binding proteins [16].

There are many causes of elevated insulin concentrations, and genetic variation in the insulin gene (*INS*) is one of the most important. The insulin gene is located on chromosome 11 (11p15.5) between the genes for tyrosine hydroxylase and insulin like growth factor 2 (IGF-2) (Figure 1). The variable number of tandem repeats (VNTR) region, located in the proximal promoter, 596 bp from translational start site of *INS*, is composed of tandemly repeated sequences of 14 – 15 bp and is supposed to have direct effects on *INS* regulation [17]. The repeat includes a variable number of the repeating oligonucleotide sequence ACAGGGGT (G/C) (T/C) GGGG. The polymorphism of the VNTR can be classified into three groups. The small class I alleles (26 – 63 repeats; average size of 570 bp) and the large class III (141 – 209 repeats, average size of 2 200 bp) alleles have reported frequencies among Caucasians of between 0.67 – 0.72 and 0.28 – 0.33 respectively, the intermediary class II (64 – 140 repeats, average size 1 200 bp) allele is rare among Caucasians [18, 19]. The extensive polymorphism of *INS*-VNTR is a result of variation in both the repeat number and sequence.

The *INS*-VNTR polymorphisms are linked with a susceptibility to a variety of disease. Class I allele homozygosity was associated in a recessive way with increased risk of developing 1TDM, while the class III alleles has been associated dominantly with the protection against developing 1TDM [20]; moreover, they were associated with increased risk of 2TDM, polycystic ovarian syndrome (PCOS), gestational DM, and with a larger weight at birth [21, 22, 23, 24, 25, 26, 27]. Results of recent studies which dealt with the relation of *INS*-VNTR to 2TDM are inhomogeneous. A comparison of 5 studies in Caucasian population showed a small risk of 2TDM occurrence in subjects bearing class III *INS*-VNTR. Although association studies failed to demonstrate a significant relation of *INS*-VNTR variants to 2TDM, an increased transmission of class III *INS*-VNTR from fathers to diabetic probands as well as an increased risk of PCOS with parental transmission of class III allele was demonstrated accordingly [20, 28]. Moreover, the association was described between the *INS*-VNTR genotype and the earlier onset of Alzheimer's disease that was independent of apolipoprotein E polymorphisms [29].

Polymorphism of *INS*-VNTR is connected to alterations in insulin expression in pancreas, thymus, placenta and lymphocytes [17, 30, 31, 32]. The *INS*-VNTR influences insulin expression both *in vitro* and *in vivo*; the class I allele is associated with increased expression of insulin mRNA and insulin levels. Young obese subjects homozygous for class I *INS*-VNTR secrete more insulin than subjects with other genotypes [27]. It is known that class I alleles consists of subclasses IC and ID [33]. The homozygosity ID/ID of class I appears to be responsible for increased insulin concentrations previously

attributed to the whole *INS*-VNTR class I [33]. In cases of massive obesity, this genotype could compensate insulin resistance associated with obesity by increased insulin secretion [33]. The *INS* gene revealed approximately 20 known haplotypes in Caucasians [34]. In Caucasians, the entire *INS*-VNTR class I allele is in the almost-complete linkage disequilibrium with neighboring *-23Hph*I "A" allele and class III with "T" allele [21], that allows this SNP to be used as a surrogate marker for *INS*-VNTR polymorphism.

It appears that polymorphism of -23*Hph*I could be a key that influences alternative splicing of intron 1. The presence of "A" allele in DNA sequence of the *INS* resulted in an increased production of mature transcript with a long 5´ leader in several cell lines, and the extended mRNA generated more pro-insulin than natural transcript [35].

Polymorphism of -23*Hph*I (replacement of thymine with adenine in the 2401 position; genetic map of V00565) [26] in the promoter of *INS* revealed a tight linkage disequilibrium with VNTR. In Europeans, the entire *INS*-VNTR class I allele is in an almost-complete linkage disequilibrium with the neighboring *-23Hph*I "A" allele and class III with "T" allele [21], that allows these single-nucleotide polymorphisms (SNP) to be used as a surrogate marker for *INS*-VNTR polymorphism in association studies.

To our knowledge, the relationship between *INS*-VNTR (and SNP *-23Hph*I A/T) and PC has not been described yet. In this study, we analyzed the association between the *INS*-VNTR polymorphism and PC, and 2TDM (respectively) as well as the influence of DM duration on the onset of PC.

The aim of this study was to analyze frequencies of "A" and "T" alleles, and their genotypes, respectively, of *INS*-VNTR in PC, health controls, and 2TDM. Moreover, we tried to elucidate relationships between individual genotypes of *-23 Hph*I SNP and the stage of PC as well as of duration of DM.

Patients and methods

Subjects. A group of 153 Caucasian subjects (86 men, 67 women) was recruited from outpatients, who were subsequently examined (from October 2005 until April 2007) at the Gastroenterological and Lipid Clinics of the Fourth Department of Medicine, Charles University in Prague. The entire group consisted of 57 (31M/20F) control subjects (age 53 ± 8 years), 45 (29M/16F) patients with DM (age 63 ± 10 years) and 51 (31M/20F) patients with PC (age 66 ± 11 years). All subjects were completely examined clinically, biochemically and genetically. The study protocol was approved by the Joint Ethical Committee of the General Teaching Hospital and the 1st Faculty of Medicine of Charles University in Prague.

Diagnosis of PC was based on the known and recommended diagnostic procedures of the American Joint Committee on Cancer (AJCC) using helical computed tomography, endoscopic retrograde cholangiopancreatography, endoscopic ultrasonography (EUS) and nuclear magnetic resonance imaging [36, 37]. The diagnosis of PC was based on bioptical (25 cases), peroperative (20) or necropsy (6 cases) examination. Diagnosis of PC was confirmed histologically in all of patients (based on histological examination of pancreatic resection or percutaneous EUS-guided aspiration cytology). Staging of PC was performed according to TNM System and the AJCC staging of PC [38].

Diabetes mellitus as well as stages of abnormal glucose metabolism (AGM) were diagnosed according to WHO criteria [39]. The control group consisted of 27 healthy persons (medical staff and students of the 1st Faculty of Medicine), 16 subjects suffering from musculoskeletal apparatus disorders (of degenerative origin) and 14 patients observed for functional disorders of gastrointestinal tract.

DNA isolation and genotyping. The isolation of DNA was performed according to standard desalting procedures [40]. The *INS* gene revealed approximately 20 known haplotypes in Caucasians [34]. In this study, we genotyped the *-23Hph*I SNP as a surrogate marker for class I and class III alleles of *INS*-VNTR gene. For amplification of the region containing -23*Hph*I polymorphism, primers according to Lindsay [26] were used. The PCR product (311 pairs of bases, bp) was digested by *Hph*I restriction enzyme. The investigated polymorphism is located in the position 2401 of DNA sequence (accession #V00565), in which thymine (T) is replaced by adenine (A) and a restriction site occurs (3´CCACT 5´). After cleaving of PCR fragment we received fragments of 144, 126 and 41 bp for the genotype A/A and 167, 144 bp for the genotype T/T. Forward and reverse primers were further used for cycle sequencing to verify the results of the PCR-RFLP method. Sequencing was performed using an automated DNA capillary sequencer (Model SEQ 8000, supplied by Beckman Coulter).

Statistics. All data were processed and statistical analyses performed in the statistical environment R, version 2.5.1. [41]. Categorical data were summarized in absolute and relative frequencies; continuous data were summarized as a mean and standard deviation. All genotype distributions were tested for Hardy-Weinberg equilibrium using an appropriate χ^2-test. Pearson χ^2-test was employed in testing for differences in the distribution of genotype frequencies in respective groups (Yates´ correction for small numbers in 2x2 tables or Monte Carlo simulation in larger tables). The statistical significance was defined as $p < 0.05$; Bonferroni´s correction was used when multiple comparisons were carried out.

Results and Discussion

Staging of PC patients is given in Table 1. Among the PC group, the unresectable disease (Stage IV) was the most prevalent, it reached nearly 54 %. In the overall PC group, 45 persons (88 %) were diagnosed with abnormal glucose metabolism (AGM) (either DM or impaired fasting glucose). In 31 cases

Table 1. TNM staging and disorders of abnormal glucose metabolism in pancreatic cancer group

AJCC * staging	stage I	stage II	stage III	stage IV	total
number of cases	-	9	14	28	51
normal fasting glucose	0	2	3	1	6
abnormal glucose metabolism (AGM) **	0	7	11	27	45
duration of AGM (< 3 years)	0	4	7	20	31
duration of AGM (> 3 years)	0	3	4	7	14

*/ American Joint Committee on Cancer

**/ Sum of patients with known DM and impaired fasting glucose

Table 2. Distribution of individual genotypes of -23*Hph*I insulin gene polymorphism among pancreatic cancer, diabetics, and controls

	genotype				allele frequency[x]		HWE	c^2 test[y]
studied group	AA	AT	TT	Total	- A	- T		
controls	21 (0.37)	26 (0.46)	10 (0.18)	57	68 (0.60)	46 (0.40)	0.3078	*
diabetes mellitus	24 (0.53)	18 (0.40)	3 (0.07)	45	66 (0.73)	24 (0.27)	0.1212	0.0218
pancreatic cancer	34 (0.67)	14 (0.27)	3 (0.06)	51	82 (0.80)	20 (0.20)	0.6441	*
stage I	-	-	-	0	-	-	-	0.018
stage II	3 (0.33)	6 (0.67)	-	9	12 (0.67)	6 (0.33)	-	
stage III	12 (0.80)	3 (0.20)	-	15	27 (0.90)	3 (0.10)	-	
stage IV	19 (0.70)	5 (0.19)	3 (0.11)	27	43 (0.80)	11 (0.20)	-	

Values represent number of cases and percentage (in parentheses) relating to individual genotype among studied groups.

Abbreviations used: PC – pancreatic cancer, DM – diabetes mellitus; CON – controls, HWE – Hardy-Weinberg equilibrium.

[x] assuming HWE

[y]/ Pearson's χ^2 test for differences between the groups

*– Comparisons between individual groups (Bonferroni correction for level of statistical significance P = 0.05/3): PC to CON: P = 0.0063 – statistically significant at 5% level;

(69 %) from those 45 subjects the duration of AGM lasted less than 3 years (Table 1).

Distribution of individual genotypes and allele frequencies of -23*Hph*I *INS* polymorphism among PC, 2TDM, and controls are shown in Table 2. There is a statistically significant difference in frequencies of individual genotypes (notably AA genotype) between the three groups (P = 0.0218). Moreover the genotype frequency differs significantly between the PC group and controls (P = 0.0063; P< 0.05/3). On the other hand, the differences in AA genotype frequencies between the PC group and 2TDM as well as between the 2TDM group and controls did not reach a statistical significance (Table 2).

Table 2 also presents the distribution of individual genotypes in relation to tumor staging. There was found a statistically significant effect of *-23HphI* A/T polymorphism on tumor staging (P=0.018). Validity of the Hardy-Weinberg equilibrium was tested in each group separately. Our data show that all genotype frequencies in the individual groups studied (PC, 2TDM, and healthy controls) are compatible with Hardy-Weinberg equilibrium (Table 2).

Relationships between *INS*-VNTR variants and PC have not been studied yet. To our knowledge, this is the first study to evaluate the relationship between the *INS*-VNTR polymorphism and the risk of PC. We have found that that the genotype A/A of *-23 Hph*I in the promoter of *INS* (and implicated class I of *INS*-VNTR) was positively associated with PC and may play a role in pancreatic carcinogenesis as well as with tumor staging.

The dismal prognosis of PC is a result of its extremely aggressive (biological) behavior and failure in early diagnosis. All over the world the incidence of PC nearly equals its mortality and less then 5 % of patients survive 5 years after diagnosis of the disease [4, 42]. The findings of our study are in agreement with overall clinical experience with staging of PC at time of its diagnosis. Prevalence of tumor stages of patients with PC, categorized according to TNM Classification [38], revealed a majority of cases in late tumor stages III and IV that contributed by 82 % cases to the whole PC group.

Therefore an early detection and primary prevention of PC remain an important medical problem. Assessments of molecular-genetic alterations in combination with the occurrence of a predisposing disease such as DM could contribute to select subjects in higher risk of PC development.

The complex relationships between disorders of glucose homeostasis and PC have long been recognized. It was found that about 80 % of PC patients have DM or glucose intolerance [43]. The data available today suggest that DM can be both a long-standing cause of PC and an early manifestation of the disease. A meta-analysis of 30 epidemiologic studies found a close relationship between previous DM (with duration at least 5 years) and subsequent development of PC [44]. A large cohort follow-up study found that DM was associ-

ated after 16 years with increased risk for many cancers including PC [45]. Similarly, a 10-year prospective conducted in Korea found an association between fasting serum glucose (and implicated DM) and many major cancers including PC [7]. Another recent meta-analysis of 17 case-control and 19 cohort (or nested case-control) studies of the association between DM and PC reported a combined age- and sex-adjusted odd ratio (OR) of 1.82 for PC among diabetics, whereas subjects in whom DM had recently been diagnosed (< 4 years) had a 50% greater risk of PC compared with individuals who had DM for 5 years and longer (OR 2.1 vs 1.5; P = 0.005) [9]. A Finnish case-cohort prospective study supports a positive association between PC risk and concentrations of glucose and insulin, as well as insulin resistance [10]. Likewise gestational DM is supposed to be a risk of PC; it usually precedes the detection of PC by 14 – 35 years [46].

On the other hand, there is growing evidence suggesting that PC causes diabetes. The majority of DM associated with PC is diagnosed either concomitantly with the diagnosis of malignancy [47], or during the 2 – 3 years before cancer detection, whereas the signs associated with PC diagnosis included younger age and the presence of gastrointestinal symptoms [48]. Short duration of DM and insulin use may reflect insulin resistance caused by PC [49]. Diabetes mellitus associated with PC may be atypical with regard to the lack of family history of DM and absence of obesity [13]. Clinical experience suggests that pancreatic tumors are causally related to insulin resistance and DM seen in PC patients, because after tumor resection the insulin sensitivity and overall diabetic state markedly improved [49]. A specific mediator that induces DM in PC has not been characterized, but amylin and 2030 mw peptide are considered to be putative diabetogenic factors [50].

In our study, the PC group consisted of 12 % subjects with normal fasting glucose and 88 % individuals with AGM [including as DM as well as impaired fasting glucose (IFG)]; the majority of them (61 %) were subjects with new-onset (< 3 years duration) AGM. Our findings concerning DM (and AGM respectively) are consistent with other studies describing the association between new-onset DM and increased probability of PC detection within 3 years meeting criteria for DM [48, 49, 51].

To our knowledge, this is the first study to evaluate the relationship between the *INS*-VNTR polymorphism and the risk of PC. Relationships between *INS*-VNTR variants and PC have not been studied yet. The first published report that was related to association between *INS* gene and risk of cancer was published by Ho Gy [52]. The class I of *INS*-VNTR was reported to be associated with prostate cancer, and to be implicated in the etiology of prostate cancer; moreover, there was a link of class I *INS*-VTNR with late-age onset of low-grade prostate tumors, categorized according to Gleason score [52].

We found that that genotype A/A of *-23 Hph*I in the promoter of *INS* (and implicated class I of *INS*-VNTR) was positively associated with PC and may play a role in pancreatic carcinogenesis. We supposed that the association between class I *INS*-VNTR variant and increased PC risk could be mediated through changed insulin levels. Limitation of this study is lack of fasting blood samples and hence, advanced biochemical analytes [insulin and other insulin like growth factors (IGF)] were not available. Insulin and IGF, which includes two ligands (IGF-1, IGF-2), membrane receptors (IGF-1R, IGF-2R), binding proteins (IGFBP) and IGFBP proteases, has been implicated in carcinogenesis. This system regulates cell proliferation, differentiation, apoptosis as well as cell transformation [53]. It was proved that increased insulin levels, due to insulin resistance in peripheral tissues, might lead to the activation of receptors of "insulin-like" growth factors, which are known to have a promoter growth effect and can thus cause a starting of malignant pancreas transformation. Pancreatic cancers are accompanied by increased expression of receptors of "insulin-like" growth factors, including insulin, IGF-1, and IGF-2.

The role of insulin in the etiology of PC is implicated from the epidemiological studies that found a positive correlations between insulin levels, glucose concentrations, insulin resistance and the risk of various major cancers, such as colon, breast, endometrial cancer as well as PC [7, 45, 54, 55]. Findings from a Swedish population-based cohort prospective study support positive relationships of obesity, intra-abdominal accumulation of fat and DM with PC risk [6]. Moreover, there are relationships between *INS*-VNTR allelic variations, *INS* expression as well as its production. Last but not least there are associations between *INS-VNTR* and *IGF2* gene, which flank the 3´ end of *INS*. It is supposed that *INS*-VNTR may act as a control element affecting the expression of both *INS* and *IGF2* [32]. It was described that *INS*-VNTR variants affect IGF-2 transcription in human placenta *in vivo*, as well as in HepG2 hepatoma cell line *in vitro* [32].

In conclusion, the presence of the "A" allele of *-23Hph*I polymorphism (and implicated class I of *INS*-VNTR) may play a role in PC development as well as in tumor staging. The class I allele of *INS*-VNTR polymorphism can thus be considered as a risk factor included in PC carcinogenesis, especially in the interaction with other genetic, metabolic, nutritional and other specific factors.

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