

Detection of viral DNA sequences in sporadic colorectal cancers in relation to CpG island methylation and methylator phenotype

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Abstract

There is evidence that insertion of viral DNA into a mammalian genome can lead to alterations of methylation patterns. The aim of the present study was to examine the presence of DNA sequences of five human DNA viruses (assessed by PCR): JC polyoma virus (JCV), human adenovirus (AdV), Epstein-Barr virus (EBV), Kaposi sarcoma-associated herpesvirus (KSHV/HHV8) and human papillomavirus (HPV) in a cohort of 186 sporadic colorectal cancers (CRCs) and related these data with the methylation status of six CIMP-specific genes (*MLH1*, *CACNA1G*, *NEUROG1*, *IGF2*, *SOCS1*, *RUNX3*) and seven cancer-related genes markers (*p16*, *MINT1*, *MINT2*, *MINT31*, *EN1*, *SCTR* and *INHBB*) assessed by methylation-specific PCR in 186 and 134 CRC cases, respectively. The AdV, KSHV and HPV were detected in 4 (2%), 2 (1%) and 0 CRC cases, respectively and thus were excluded from further analyses. Although, 19% and 9% of the CRCs were positive for EBV and JCV respectively, no associations between virus presence and CpG island methylation were found after correction for multiple testing. Our results demonstrate that the presence of DNA sequences of JCV and EBV in CRC is unrelated to the methylation of the 13 cancer-related CpG islands and CIMP.

Background

The process of DNA methylation in human cells are controlled by DNA methyltransferases (DNMTs). DNMTs catalyze the transfer of a methyl group from the

methyl donor to the 5' position on the cytosine ring [1]. CpG island methylation is prevalent in human sporadic cancers resulting in the transcriptional silencing of many important genes [2]. Abberant CpG island methylation is one of the hallmarks of sporadic CRC and in this context it has been observed that a subset of CRCs exhibit an exceptionally high frequency of methylation of discrete CpG islands referred to as the CpG island methylator phenotype (CIMP) [3]. Although the mechanism responsible for abberant CpG island methylation and/or CIMP has not yet been elucidated, there is mounting evidence that infectious agents, such as DNA viruses, may cause aberrant methylation in CRCs [4].

Evidence that DNA viruses influence aberrant methylation comes from the observation that insertion of adenovirus (AdV) DNA into a mammalian genome can lead to alterations of methylation patterns in cellular DNA [5]. Moreover, a two recent studies have reported that Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines demonstrate alterations of methylation patterns when compared to peripheral blood leukocytes [6,7]. Finally, abberant methylation of p16 tumor suppressor gene has been reported in Kaposi sarcoma-associated herpesvirus (KSHV/HHV8) associated primary efusion lymphoma. It was also demonstrated repeatedly that various viral proteins interact with a number of host epigenetic regulators which may contribute to alternations in DNA methylation and histone modifications [8]. The AdV small E1A protein binds to DNA methyltransferase 1 (DNMT1), increases its activity and alters global patterns of histone modification [9]. The human papillomavirus type 16 (HPV16) E6 oncoprotein interacts with and thus inhibits the histone acetyltransferase CBP/p300 complex, whereas the E7 oncoprotein binds to the histone deacetylase complex Mi2 β [10]. The KSHV (HHV8) LANA protein activates DNMT3a facilitating *de novo* promoter methylation [11]. The EBV latent membrane protein 1 (LMP1) activates all three human DNMTs, which increases *de novo* promoter methylation [12]. Although none of the JC virus (JCV) proteins have been reported to interact with any epigenetic regulator, some

previous reports have suggested a link between expression of the JCV T-antigene and extensive promoter methylation leading to methylator phenotype (CIMP) in colorectal cancer (CRC) [13].

In this study we assess the presence of DNA sequences from five viruses (JCV, EBV, AdV, KSHV (HHV8) and HPV) in CRCs in relation to methylation of a number of cancer-related genes as well as to CIMP.

Methods

Patients. The study was performed on 186 surgically resected frozen tissues of sporadic CRCs that were obtained between 2001 and 2008 from the 2nd Department of General and Oncological Surgery, Wrocław Medical University and the Department of General Surgery, Regional Specialized Hospital, Wrocław.

The CRC patient's group consisted entirely of Polish individuals (all Caucasians). Only patients with primary, sporadic colorectal cancer who had not received preoperative therapy were included into the studies. Informed consent was obtained from all patients. The study was accepted by the Wrocław Medical University Ethics Committee.

BRAF V600E mutation. Detection of *BRAF* V600E in tumor tissues was carried out using a procedure originally described by Sapio et al. [14]. Briefly, mutant allele-specific PCR was used to amplify the exon 15 region of the *BRAF* gene. PCR products with an expected size of 125 bp were resolved on a 2.5% agarose-ethidium bromide gel.

Bisulfite Treatment of DNA, Methylation-Specific PCR and CIMP. Bisulfite treatment of genomic DNA obtained from resected frozen tumor tissues was carried out using a procedure described by Chan et al. [15]. Approximately 50 ng of the modified DNA was amplified in a PTC 200 DNA Engine Thermal cycler (MJ Research, Inc. Waltham, MA) with primers specific to either the methylated or unmethylated promoter sequences of the *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, *SOCS1*, *hMLH1*, *p16*, *MINT1*, *MINT2*, *MINT31*, *EN1*, *SCTR* and

INHBB genes. The primer sequences and amplification conditions of methylation-specific PCR utilized in this study are described elsewhere [3,15,16]. CIMP was defined by the use of a specific panel of markers and criteria described by Weiseberger et al. [3]. Briefly, after the analysis of the methylation of a panel of 5 markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*), CIMP+ tumors were defined as those with at least 3 methylated CIMP markers. CIMP- tumors were defined as those with at most 2 methylated CpG islands.

Viral DNA amplification. Firstly, all samples were examined for DNA integrity using amplification of the β -globin gene. Great care was taken to avoid specimen contamination during the course of this investigation. Sample preparation, electrophoresis and PCR solution preparation were conducted in physically separated laboratory areas. The viral sequences were amplified using a single PCR approach to avoid false-positives and ensure reliable PCR detection. For JCV, KSHV (HHV8) and EBV we used species-specific PCR primers [13,17]. As the HPV and AdV are species with a high degree of genetic heterogeneity, we used broad-spectrum PCR primers that permit the simultaneous amplification of a range of HPV or AdV types in a single PCR test [18,19]. A negative control was included in each PCR assay. All samples were reexamined for the presence of viral DNA. All positive samples for EBV and JCV were confirmed using restriction enzyme digestion.

All PCR reactions were carried out separately in a 25 μ l reaction volume containing 1x PCR buffer (Qiagen), 1.5 mmol/L MgCl₂, 200 μ mol/L deoxynucleoside triphosphate, 50 ng of genomic DNA, 0.2 μ mol/L of each primer, and 0.75 units of Hot-Start DNA polymerase (Qiagen). All PCR reactions were run under the conditions described in the references, except for an initial denaturation period of 15 min at 95°C, which we applied in all tests. Primer sequences and related references are shown in Table 1. All positive controls were purchased from Advanced Biotechnologies Inc (Columbia, Maryland) except of HPV, where DNA obtained from larynx tumor positive for HPV 16 was applied (see Table 1).

Statistical analysis. The Pearson chi-squared test (if all expected cell frequencies were ≥ 5) or Fisher's exact test was used to test whether the presence of a virus is associated with the methylation of a CpG island or CIMP status. All p -values were two-sided and Bonferroni correction was taken into consideration because of the multiple comparisons carried out in this study. The R statistical package was used to carry out the necessary statistical tests and calculate the confidence intervals for the odds ratio.

Results

Study group and CIMP classification. We examined the CIMP status of 186 sporadic CRCs by methylation-specific PCR using a CIMP specific marker panel (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*) [3]. The characteristics of study group, both overall and with respect to CIMP status, are shown in Table 2. On the average, the CIMP+ cases tended to be older than CIMP- cases, however, this differences did not attain statistical significance. There were no significant differences between CIMP- and CIMP+ cases in sex distribution. Since CIMP was defined as the presence of at least 3 methylated sites out of the 5 studied loci, 25% (46/186) of the tumors were classified as CIMP+. A strongly bimodal distribution of tumors according to the number of methylated loci was observed (Figure 1). To examine whether our CIMP classification was adequate, we determined the presence of the *BRAF* V600E mutation and *MLH1* methylation. We observed a significant association of both the *BRAF* V600E mutation and *MLH1* methylation with CIMP+ tumors (OR = 12.33, 95% CI = 4.21-41.54; $P = 1.8 \times 10^{-7}$ and OR = 15.5, 95% CI = 4.54-68.65; $P = 2.8 \times 10^{-7}$, respectively). These results, together with the strongly bimodal distribution of the number of methylated tumors in our cohort proved that our CIMP classification was appropriate.

Detection of viral DNA sequences in colorectal cancers. In the current study, we analyzed 186 cases of sporadic colorectal tumors (all amplifiable with a β -globin primer set) for the presence of five viruses (JCV, AdV, EBV, KSHV (HHV8) and HPV) using PCR. The AdV,

KSHV and HPV were detected in 4 (2%), 2 (1%) and 0 CRC cases, respectively and thus these species were excluded from the statistical analyses. 19% (36/186) cases were positive for EBV and 9% (17/186) of the cases were positive for JCV. There was no relationship between the presence of EBV and JCV and sex (see Table 3).

Relationship of JCV and EBV with CIMP and methylation in CIMP markers. The overall results on the presence of EBV and JCV according to CIMP and methylation of CIMP-specific markers are presented in Table 3 and Figure 1. The presence of JCV was significantly less common in tumors with at least one methylated CIMP specific marker (5.6% (7/124); $P = 0.03$) than in the group without methylated CIMP specific markers (16% (10/62)), Figure 1. We also examined the relationship of EBV and JCV with methylation for each CIMP specific marker individually (Table 3). In this analysis EBV was inversely associated with methylation of *RUNX3* ($P = 0.048$) and JCV was inversely associated with methylation of *CACNA1G* ($P = 0.03$). However, these associations became nonsignificant after correction for multiple testing.

Relationship of JCV and EBV with methylation in individual CpG islands. Because JCV and EBV have been implicated in CpG island methylation, we examined the relationship between the presence of these two species and methylation in 7 individual CpG islands (Table 4). For this analysis we were able to use 134 of the 186 CRCs due to the limited availability of DNA from the tumors. In this analysis JCV showed an inverse association with methylation of *MINT1* ($P = 0.04$). However, this association became nonsignificant after correction for multiple testing

Conclusions

Epigenetic alternations play a key role in the process of colorectal carcinogenesis [20]. The recently described CpG island methylator phenotype (CIMP) in sporadic CRC is an alternative tumorigenesis pathway characterized by the methylation of multiple promoter regions of tumor suppressor genes harboring CpG islands [3]. Paradoxically, despite dozens of studies, the defects in the methylation machinery leading to CIMP in CRC remain still unidentified [4].

Several lines of evidence suggest that a number of viral proteins interact with the host's epigenetic machinery, most likely to evade detection by the host's immune system [8]. Moreover, experimental approaches, such as transfection of mammalian cells with an adenovirus, demonstrate that insertion of viral DNA can alter the host's DNA methylation patterns regardless of the transcription state of the viral genome [5,21]. Further evidence of virus-induced alternations in host's DNA methylation patterns was observed in EBV transformed lymphoblastoid cells [6,7]. Therefore, the DNA viruses such as JCV, AdV, EBV, KSHV (HHV8) and HPV seemed to be reasonable candidates for being a causative agents of alterations in DNA methylation resulting in CIMP in sporadic CRC.

In the present study AdV and KSHV (HHV8) were detected in 4 and 2 CRC cases, respectively. Hence, these species were excluded from further analyses. Of note, the very low frequency of infections in our CRC group by these species are in agreement with those reported by Knösel et al. [22], who studied various infectious pathogens in Crohn's disease.

In our CRC group no positive signals (0/186) were obtained for HPV. HPV DNA has been detected in CRCs by others with a frequency ranging from 50% to 80% [23-25]. Interestingly, in the all of above-mentioned reports a nested PCR was employed to detect HPV. In our opinion, this detection technique is likely to introduce a high rate of false positives or the detection of insignificant virus load in the sample, which leads to overestimation of virus presence in the sample, therefore, we decided to stay in line with a single PCR approach for

all virus species investigated in this study. In agreement with our strategy, the results of Atula et al. [26] suggest that HPV DNA detected by nested PCR in laryngeal carcinoma cell lines is likely to relate to the presence of minimal amounts of HPV (20 HPV-positive cells among 10^6 tumor cells) suggesting non-clonal persistence of HPV in laryngeal carcinomas. Most importantly, we were able to amplify HPV DNA from larynx cancer samples by our PCR approach (data not shown) which further suggests lack or minimal amounts of HPV copies in our CRC cohort.

Of the other viruses examined, EBV was the most frequent pathogen (19% of the cases). Few papers have been also published on the presence of EBV in CRCs, moreover with contradicting results. EBV DNA has been detected in CRCs with a frequency ranging from 0% to 19% [27-29]. In great majority of above-mentioned studies EBV has been detected by using *in situ* hybridisation of the small EBV-encoded RNA1 (EBER1) which is believed to be consistently expressed in EBV infection. However, an EBER-negative form of EBV infection has been observed in breast cancers and hepatocellular carcinoma and therefore a lack of detectable EBNA1 expression cannot be taken as proof of absence of the virus [30,31]. Since in the present study we used DNA obtained from resected tumors, it is possible that some of the EBV positive signals reflect the presence of tumor-infiltrating lymphocytes (TILs) in the tumour stroma that carry EBV rather than the presence of EBV-positive tumor cells. However, Yoshiyama et al. [32] and others reported that EBV is able to infect epithelial cell lines *in vitro* only by co-culture with EBV-infected B cells, therefore the contribution of EBV-positive TILs (as possible EBV transmitters) in pathogenesis of colon cancer can not be completely excluded [33].

In general, EBV was not associated either with the CIMP phenotype or methylation of any of the individual CpG islands studied, except for an inverse association with methylation

in one of the CIMP specific markers (*RUNX3*) (nonsignificant after correction for multiple testing).

The second most frequent virus in our CRC group was JCV (9% of the cases). As in the case of prevalence of HPV in CRCs, the papers published on the presence of JCV T-antigen sequence in CRCs display contradictory results with frequencies ranging from 0% in Italian and Spanish study, through 26% in Japanese cases to 77% in American patients [13,34,35,36]. Given the all above-cited studies based on relatively similar PCR approaches the discrepancies of JCV frequencies in CRC may reflect ethnic-dependent epidemiology of JCV or lack of reliable and reproducible test for the detection of JCV DNA [37]. A previous study by Goel et al. [13] reported an association between JCV T-antigen expression and methylation of the promoter region of various cancer-related genes in colorectal cancer. Goel et al. [13] also hypothesized that the JCV T-antigen may be responsible for induction of the methylator phenotype in CRC. Contrary to these results, we found that the presence of JCV T-antigen sequence was less common in tumors with at least one methylated CIMP specific marker. Moreover, we showed that among the CRC cases the presence of JCV T-antigen sequence was inversely associated with methylation in two CIMP-related genes (*CACNA1G* and *MINT1*). Although these associations became nonsignificant after correction for multiple testing it did not escape our attention that the presence of JCV T-antigen sequence show some tendency towards unmethylated CpG islands. Similar results were presented in a very recent paper by Nosho et al. [38], who observed an inverse association of JCV T-antigen expression with CIMP and a lack of association with methylation in 16 genes in a large sample of CRCs. Interestingly, some recent reports show very strong evidence that JCV T-antigen interacts with p53 and Rb family proteins and therefore may induce chromosomal instability (CIN), which is a largely independent from the CIMP pathway in colorectal carcinogenesis [39,40]. The independence of the CIN and CIMP pathways in sporadic CRC manifests itself via the high

level of chromosomal aberrations in tumors with none of the CIMP markers methylated [41]. The more common presence of JCV in a group of CRCs without any CIMP marker methylated revealed by our study provides some support to the notion that JCV may contribute to CIN in a fraction of CRC cases [38].

A potential limitation of this study is that DNA methylation investigation was limited to several CpG islands, therefore the influence of presence of JCV and EBV on DNA methylation in CRC needs to be further elucidated on the genome-wide scale. Finally, further research is needed to assess the relationship between virus load and DNA methylation in CRC.

In summary, our study provides no evidence of involvement of AdV, KSHV (HHV8) and HPV in pathogenesis of CRC in Polish population. The presence of JCV and EBV sequences in CRCs was not related to methylation of the 13 cancer-related CpG islands and CIMP.

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Figure 1. Bimodal distribution of the number of methylated CIMP-specific markers in 186 colorectal tumor specimens with relation to EBV and JCV presence

Table 1. Primer sequences used in this study

| | Primer sequences (5'→3') | Target sequence | Product size | Positive control | Reference |
|----------------|---|-----------------------|--------------|-------------------------------------|-----------|
| β-globin | GlobinF ACACAACCTGTGTTCACTAGC GlobinR GGAAAATAGACCAATAGGCTG | β-globin | 225bp | | (17) |
| EBV | EBVF GATTTGGACCCGAAATCTGAT EBVF TCTGGGGGCTTATTCCTCTT | EBV BamHI W repeat | 201bp | EBV B95-8 Strain | (17) |
| KSHV (HHV8) | ORFK9F GTCTCTGCGCCATTCAAAC ORFK9R CCGGACACGACAATAAGAA | vIRF-1 | 184bp | KSHV/HHV-8 KS- 1 Strain | (17) |
| AdV | Hex3 GACATGACTTTCGAGGTCGATCCCATGGA Hex4 CCGGCTGAGAAGGGTGTGCGCAGGTA | hexon gene | 139bp | Adenovirus-2 Infected Cell DNA | (19) |
| HPV | HPVpU- M TGTCAAAACCGTTGTGTCC HPVpU-31B TGCTAATTCGGTGCTACCTG HPVpU-2R GAGCTGTCGCTTAATTGCTC | E6 and E7 regions | 228~268 bp | HPV-16 positive larynx tumor DNA | (18) |
| JCV | JCVF ATGTATTCCACCAGGATTCCCATTATC JCVR AGTTCTTGGAGACACCCCCTACAG | large T antigen | 154bp | JCV MAD1 Strain | (13) |

Table 2. Characteristics of CRC patients and control subjects

| Variable | All CRCs (n=186) | CIMP+ CRCs (n=46) | CIMP - CRCs (n=140) | <i>p</i> -value |
|------------------------|---------------------|----------------------|------------------------|--|
| Age (SD ^a) | 65 (±10) | 68 (±11) | 64 (±10) | ^b2.8×10⁻⁷ |
| Male (%) | 105 (56) | 28 (61) | 77 (56) | |
| Female (%) | 81 (44) | 18 (39) | 63 (44) | |
| hMLH1 (%) | | | | |
| Positive | 19 (10) | 15 (33) | 4 (3) | |
| Negative | 167 (90) | 31 (67) | 136 (97) | |
| BRAF V600E (%) | | | | ^b1.8×10⁻⁷ |
| Positive | 23 (12) | 17 (37) | 6 (4) | |
| Negative | 163 (88) | 29 (63) | 134 (96) | |
| Right-sided | 24 (21) | 10 (37) | 14 (18) | ^c0.02 |
| Left-sided | 85 (79) | 17 (63) | 68 (82) | |
| No info | 77 | | | |

^aSD - standard deviation

^b*p*-value refers to Fisher's exact test

^c*p*-value refers to Pearson's chi-squared test

Table 3. Frequency of JCV and EBV in 186 colorectal cancers in relation to CIMP and methylation of CIMP markers

| Marker | Total | EBV+ (%) | EBV- (%) | <i>p</i>-value | JCV+ (%) | JCV- (%) | <i>p</i>-value |
|--------------------|--------------|-----------------|-----------------|-----------------------|-----------------|-----------------|-----------------------|
| | 186 | 36 (19) | 150 (81) | | 17 (9) | 169 (91) | |
| male | 105 | 18 (17) | 87 (83) | 0.46 | 10 (10) | 95 (90) | 1.00 |
| female | 81 | 18 (22) | 63 (78) | | 7 (9) | 74 (91) | |
| CIMP + | 46 | 6 (13) | 40 (87) | 0.21 | 3 (7) | 43 (93) | 0.57 |
| CIMP - | 140 | 30 (21) | 110 (79) | | 14 (10) | 126 (90) | |
| n CpG ≥ 1 | 124 | 24 (19) | 100 (81) | 1.00 | 7 (6) | 117 (94) | 0.03 |
| n CpG = 0 | 62 | 12 (19) | 50 (81) | | 10 (16) | 52 (84) | |
| BRAF (+) | 23 | 1 (4) | 22 (96) | 0.08 ^a | 0 (0) | 23 (100) | 0.13 ^a |
| BRAF (-) | 163 | 35 (21) | 128 (79) | | 17 (10) | 146 (90) | |
| MLH (+) | 19 | 2 (11) | 17 (89) | 0.54 ^a | 1 (5) | 18 (95) | 0.70 ^a |
| MLH (-) | 167 | 34 (20) | 133 (80) | | 16 (10) | 151 (90) | |
| CACNA1G (+) | 69 | 9 (13) | 60 (87) | 0.12 | 2 (3) | 67 (97) | 0.03 |
| CACNA1G (-) | 117 | 27 (23) | 90 (77) | | 15 (13) | 102 (87) | |
| IGF2 (+) | 44 | 5 (11) | 39 (89) | 0.19 | 4 (9) | 40 (91) | 1.00 |
| IGF2 (-) | 142 | 31 (12) | 111 (88) | | 13 (9) | 129 (91) | |
| NEUROG1 (+) | 72 | 16 (22) | 56 (78) | 0.45 | 3 (4) | 69 (96) | 0.07 |
| NEUROG1 (-) | 114 | 20 (18) | 94 (72) | | 14 (12) | 100 (88) | |
| RUNX3 (+) | 62 | 7 (11) | 55 (89) | 0.048 | 3 (5) | 59 (95) | 0.18 |
| RUNX3(-) | 124 | 29 (23) | 95 (77) | | 14 (11) | 110 (89) | |
| SOCS1 (+) | 51 | 10 (20) | 41 (80) | 1.00 | 4 (8) | 47 (92) | 1.00 |
| SOCS1 (-) | 135 | 26 (19) | 109 (81) | | 13 (10) | 122 (90) | |

^a *p*-value refers to Fisher's exact test

Table 4. Frequency of JCV and EBV in 134 colorectal cancers in relation to methylation of individual cancer-related CpG islands

| Marker | Total | EBV+ (%) | EBV- (%) | <i>p</i>-value | JCV+ (%) | JCV- (%) | <i>p</i>-value |
|-------------------|--------------|-----------------|-----------------|-----------------------|-----------------|-----------------|-----------------------|
| | 134 | 22 (16) | 112 (84) | | 13 (10) | 121 (90) | |
| p16 (+) | 44 | 8 (18) | 36 (82) | 0.80 | 2 (5) | 42 (95) | 0.22 |
| p16 (-) | 90 | 14 (16) | 76 (84) | | 11 (12) | 79 (88) | |
| MINT1 (+) | 34 | 4 (12) | 30 (88) | 0.59 | 0 (0) | 34 (100) | 0.04 |
| MINT1 (-) | 100 | 18 (18) | 82 (82) | | 13 (13) | 87 (87) | |
| MINT2 (+) | 53 | 6 (11) | 47 (89) | 0.23 | 5 (9) | 48 (91) | 1.00 |
| MINT2 (-) | 81 | 16 (20) | 65 (80) | | 8 (10) | 73 (90) | |
| MINT31 (+) | 49 | 6 (12) | 43 (88) | 0.46 | 6 (12) | 43 (88) | 0.55 |
| MINT31 (-) | 85 | 16 (19) | 69 (81) | | 7 (8) | 78 (92) | |
| EN1 (+) | 45 | 8 (18) | 37 (82) | 0.80 | 5 (11) | 40 (89) | 0.76 |
| EN1 (-) | 89 | 14 (16) | 75 (84) | | 8 (9) | 81 (91) | |
| SCTR (+) | 113 | 19 (17) | 94 (83) | 1.00 | 12 (11) | 101 (89) | 0.69 |
| SCTR (-) | 21 | 3 (14) | 18 (86) | | 1 (5) | 20 (95) | |
| INHBB (+) | 38 | 5 (13) | 33 (87) | 0.61 | 4 (11) | 34 (89) | 1.00 |
| INHBB (-) | 96 | 17 (18) | 79 (82) | | 9 (9) | 87 (91) | |