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# The prevalence of HPV infection in rectal cancer – Report from South – Central Poland (Cracow region)

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ABSTRACT

Some studies suggest that HPV infection may be important carcinogenic factor in development of some part of colorectal cancers. However, in the worldwide literature concerning this type of tumours, the great variability in HPV frequency is noticed. In Poland, the incidence of HPV infection in colorectal cancers was examined in five studies so far and their results are also conflicting. Therefore, the aim of the present study was to assess the HPV presence in the group of 120 patients with adenocarcinomas of rectum. HPV infection was assessed on the basis of DNA extracted from collected formalin fixed paraffin embedded tumour specimens. Viral presence was evaluated using two PCR based methods: nested PCR and quantitative PCR (qPCR) with primers specific for HPV16. All HPV positive samples were subjected to virus genotyping using AmoyDx® Human papillomavirus (HPV) Genotyping Detection Kit and P16 immunostaining. Among 120 evaluated colorectal tumours, HPV DNA was detected in 2 cancers (1.67%) by nested PCR and in 2 (1.67%) tumours by qPCR, including 1 sample diagnosed as HPV positive on the basis of both PCR variants. Two HPV positive cancers had HPV16 infection and other one HPV18. All three tumours with positivity of HPV DNA were P16 negative. In south – central Poland, HPV infection in rectal cancers probably has not influence on rectal carcinogenesis.

## 1. Introduction

Human papillomavirus (HPV) infection is an important etiological factor in development of a subset of anogenital tumours. Among them, the most common are cervical and anal cancers [1]. Some studies suggest that HPV infection may also promote development of colorectal cancers, mainly those localized in rectum (for review, see [2]). However, in the literature concerning this type of tumours, a great variability in HPV prevalence is noticed: from 0 [3–11] to 84.2% [12]. Even in two meta-analyses regarding this issue, both covering 53 papers, divergent results have been presented. In one of them the percentage of infected colorectal cancers was 11.9% [13], while in the second it was 31.9% [14]. There are many possible reasons for this large discrepancy: (1) geographical heterogeneity (high incidence of HPV infection in South America, Asia and Middle East *vs* low in Australia, Europe and North America), or (2) different sensitivity and specificity of HPV detection method (lower HPV incidence in non-PCR-based studies *vs* higher in analysis using PCR technique), (3) type of material which was used to assess HPV presence (higher viral persistence in fresh or frozen material *vs* lower in formalin-fixed paraffin-embedded [FFPE]), (4) date of sample collection (lower frequency before year 2000 *vs* higher after this date) [2,13,14].

In Poland, the presence of HPV infection in colorectal cancers has been examined in five studies so far and their results are conflicting (Table 1). In two of them [3,4], including together more than 200 patients from South Poland, no HPV infection was found in this type of tumours. However, in three others [15–17], covering in total 99 patients with colorectal cancers from Warsaw area and East Poland, HPV infection was detected in the range from 20% [15] to 67% [16]. What is interesting, to evaluate HPV presence, in all of these studies PCR-based methods were used; in two of them [5,17] analysis was performed on frozen tumour specimens and in others – on FFPE material [4,15,17]. Taking into account potential influence of HPV on disease prevention and patients' prognosis and inconsistent results regarding viral infection in colorectal cancers in Poland, the aim of the present study was to assess HPV status in a group of 120 patients with rectal cancer using two PCR-based methods: nested PCR and quantitative PCR (qPCR). In order to confirm HPV infection in rectal cancer, for each HPV positive

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**Table 1**
The incidence of HPV infection in colorectal cancers in Poland.

| Authors | Geographical region of Poland | Number of patients/ tumour localization | Year of sample collection | Type of sample | Method of DNA isolation | Method of HPV detection | Percentage of HPV positivity |
|---|---|---|---|---|---|---|---|
| Jarzyński et al. [15] | East | 50 11 – sigmoid colon 13 – ascending + descending colon 13 – cecum 12 – rectum 1 – colon and rectum | not given | FFPE | QiaAmp DNA Mini Kit | InnoLipa | 20.0 2 (15.4) – ascending + descending colon 2 (18.2)– sigmoid colon 3 (23.1)- cecum 3 (25.0) - rectum |
| Snietura et al. [4] | South | 30 6 – right colon 16 – left colon 18 - rectum | not given | FFPE | Not given | qPCR (real time Higher Risk test (Abbott Moleculars) + P16 (IHC) | 0.0 |
| Miąsko et al. [16] | East | 40 10 – sigmoid colon 7 – ascending colon 5 – descending colon 2 - transverse colon 2 – cecum 14 - rectum | not given | FFPE | QiaAmp DNA Mini Kit | PCR with HPV primers (228-268 bp) + ISH | 52.5 7 (70.0)- sigmoid colon 4 (57.1) – ascending colon 7 (50.0) – rectum |
| Karpiński et al. [5] | South | 186 24 - right colon – 85- left sided colon 77 - no data | 2001-2008 | frozen | QiaAmp DNA Mini Kit | PCR with HPV primers (228-268 bp) | 0.0 |
| Młynarczyk et al. [17] | Central | 9 tumour localization – no data | not given | frozen | Not given | PCR with primers for HPV16 and 18 | 67.0 tumour localization – no data |

Abbreviations: FFPE- formalin fixed paraffin embedded tissues, PCR – polymerase chain reaction, qPCR – quantitative polymerase chain reaction, bp - base pair, IHC – immunohistochemistry, ISH – *in situ* hybridization.

tumours in PCR analysis, P16 immunostainning was performed on the basis of FFPE tissue sections.

## 2. Material and methods

### 2.1. Material

The study was conducted in the group of 122 patients with rectal cancers treated between 2007–2014 in Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Cracow Branch. From these patients, on the need of the study concerning biological indicators of tumour response to preoperative radiotherapy [18], FFPE tumour samples were taken before radiotherapy (by rectoscopy) were collected. Inclusion criteria to the study were: histologically proved adenocarcinoma, locally resectable tumours, no previous radiotherapy of pelvic area. Exclusion criteria were as follows: metastatic disease, other malignant cancer, and patient refusal.

In the present study, HPV presence and genotyping was performed on the basis of DNA extracted from collected FFPE samples. Before DNA extraction, each paraffin block undergo histopathological verification in order to confirm histopathological diagnosis and grade.

The study was approved by the Ethical Committee at the Regional Medical Chamber in Cracow (Poland) on 19 September 2012 (109/KBL/OIL/2012). During the study no direct contact with patients and use of their personal data were necessary, therefore no informed consents from patients were required. All samples were anonymized.

### 2.2. DNA extraction

DNA extraction was performed for 120 tumours due to lack of cancer tissue in two paraffin blocks. For DNA extraction, 4 µm thick sections of each FFPE specimen were used (3–7 sections depending on sample size). In order to prevent cross contamination, the microtome and other accessories were cleaned with DNA Away (cat. no. 7010, Thermo Fisher Scientific, Inc., Waltham, USA) and fresh blade was installed before cutting each block. The first 2–3 sections were discarded. During cutting, excess paraffin was trimmed away, the sections were placed in 1.5 ml Eppendorf tube and spun briefly. DNA was isolated using ReliaPrep™FFPE gDNA Miniprep System (Promega, Madison, USA) based on manufacturer`s suggestions with our own modification. First, the sections were incubated in 300–500 mL (depending on the section size) of mineral oil for 1 min., at 80 °C. After adding Solution Buffer and centrifugation (12 000 rpm, 15 s) two phases were observed: aqueous and oil. Proteinase K dissolved in Lysate Buffer was added to aqueous phase and samples were incubated for the whole night at 56 °C (own modification) and then for 1 h at 80 °C. After cooling to room temperature, the samples were treated with RNAse A for 5 min. and incubated with mixture of BL Buffer and 100% ethanol. After centrifugation (12 000 rpm, 15 s) the aqueous phase was transferred to the Binding Column which was washed twice. DNA was eluted with 50 µl of Elution Buffer. Quantity and quality (A260/280 and A260/230 ratios) of isolated DNA were assessed spectrophotometrically with Biophotometer Plus (Eppendorf AG, Hamburg, Germany). Isolated DNA samples were stored at −20 °C until analysed.

### 2.3. Nested PCR

This PCR variant involves two pairs of primers - outer and inner - used in two successive PCR runs and the product of the first reaction (with outer primers) serves as a template in the second run (with inner primers).

To detect L1 gene fragment of multiple HPV types during one experiment (without indication the virus type precisely) we used PGMY09/PGMY11 [19] and GP5 + /GP6 + [20] primer sets synthesized in Genomed, Poland. During the first reaction 4 µl of DNA was amplified in a volume of 20 µl contained 4 mM of $MgCl_2$, 200 µM of each

dNTP, 0.1 μM of each PGMY primer and 1.5 U of TaqNova polymerase. Thermal cycling was as followed: initial denaturation at 94 °C for 4 min, then 40 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 30 s, and final elongation at 72 °C for 4 min. All PCR reagents except for primers were bought from DNA Gdansk, Poland. The second 20 μl reaction mixture contained 3.5 mM of $MgCl_2$, 200 μM of each dNTP, 0.6 μM of each GP + primer, 0.5 U of TaqNova polymerase and 4 μl of the product of the first reaction. Thermal cycling conditions were: initial denaturation at 94 °C for 4 min, then 40 cycles of 94 °C for 30 s, 40 °C for 30 s and 72 °C for 30 s, and final elongation at 72 °C for 4 min.

In each PCR run water was used as negative control and DNA isolated from HPV-positive cervical cancer tissue as positive control. The final products were separated electrophoretically in 2% agarose gel and visualized using SimplySafe dye (EURx, Poland). For each tumour 2 analyses were performed and samples with at least one positive result were classified as HPV positive and undergo to genotyping procedure.

### 2.4. Quantitative PCR – amplification of β-action and HPV16 presence with type specific primers

DNA extracted from FFPE samples was subject to qPCR in order to check amplification of housekeeping gene - *β-actin* and HPV16 presence by qPCR using Viia7 System (Applied Biosystems, USA).

In order to check pattern of DNA degradation, each sample was subjected to qPCR for amplification of 139 bp fragment of *β-actin* gene using TaqMan® Gene Expression Assay (Thermo Fisher Scientific, Waltham, USA), with mix of specific primers and MGB probe. Amplification was performed in reaction volume of 20 μl, containing: 10 μl of Fast Universal PCR Master Mix (2x) (Thermo Fisher Scientific, Waltham, USA), 1 μl of TaqMan Gene Expression Assay and 50 ng of DNA template. qPCR was initiated with 20 s incubation at 95 °C, then 40 cycles at 95 °C for 1 s and 60 °C for 20 s were applied (ViiA 7, Thermo Fisher Scientific, Waltham, USA). Two replicates were used per sample.

The HPV16 presence was assessed on the basis of amplification of 81 bp fragment of viral *E6* gene with primers (F: GAG AAC TGC AAT GTT TCA GGA CC, R: TGT ATA GTT GTT TGC AGC TCT GTG C) and TaqMan probe (6FAM- CAG GAG CGA CCC AGA AAG TTA CCA CAG TT-TAMRA), all synthesized by Thermo Fisher Scientific, Waltham, USA [16]. Amplification was performed according to Si et al. [14] in a 25 μl reaction volume containing: 12.5 μl of Fast Universal PCR Master Mix (2x) (Applied Biosystems, USA), 100 nM of each primer, 300 nM of TaqMan probe () and 20 ng of DNA template. Thermal cycling consisted in a step of 20 s at 95 °C, followed by 45 cycles of 3 s at 95 °C and 30 s at 60 ºC. Two replicates were analyzed. As a negative control, to each run water was added instead DNA template and as positive control serve genomic DNA (Roche) (β-actin) or Ca-Ski cell line, which cells contain around 600 integrated HPV16 copies.

### 2.5. HPV genotyping

All HPV positive samples in nested PCR or qPCR were subjected to virus genotyping using AmoyDx® Human papillomavirus (HPV) Genotyping Detection Kit (Amoy Diagnostics Co., LTD, China). It is real-time PCR based test allowing for genotyping of 19 high risk HPV (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82) and 2 low risk (6 and 11) on the basis of virus *L1* gene amplification The procedure was performed according to manufacturer`s protocol. First, reaction mixture comprising 2.7 μl HPV[21] Enzyme Mix and 42.3 μl DNA was prepared and 5 μl of this mixture was transferred to each PCR tube of 8-tube strip with 25 μl of PCR Reaction Mix with Taq DNA polymerase. One 8-tube strip, containing8 types of Reaction Mix: 7 with primers and fluorescent probes specific for definitive HPV DNA and one with internal positive control system was employed for each sample. The qPCR was carried out in ABI7500 cycler (Life Technologies) using the following conditions: stage 1: 50 °C, 2 min. and 95 °C, 5 min. (1 cycle), stage 2: 95 °C, 25 s, 60 °C, 20 s and 72 °C, 20 s (10 cycles), stage 3 95 °C, 25 s, 60 °C, 35 s and 72 °C, 20 s (31 cycles, data collection).As a negative control, to each experiment one 8-tube strip with water instead template was added. HPV genotype was determined by analysis of combination of fluorescent signals from FAM, CY5 and HEX/VIC in each tube according to manufacturer`s instruction.

### 2.6. P16 immunostaining

P16 immunostaining was performed in the subgroup of three HPV16 positive tumours using CINtec® Histology Kit (Roche, Heidelberg, Germany) according to the manufacturer`s procedure. All details regarding staining procedure was described earlier [21]. The the set of staining negative (absence of primary antibody) and positive (cervical cancer with known P16 strong reaction) controls were added. Immunopositivity was defined according to Lewis et al. [22] as follows: > 75% of positive staining cells or > 50% staining with > 25% confluent areas of positive staining.

## 3. Results

### 3.1. Patients

Among 120 patients included into the study, there were 34 women (28.3%) and 86 men (71.7%) in age ranging from 30 to 82 years (mean and median values: 61.0 years ± 0.9 and 61 years) (Table 2). In this group, male patients predominated, as well as those with tumours in clinical stage II and grade 2. Most patients (n = 113, 94.2%) were treated with short course of preoperative radiotherapy (RT) (5 x 5 Gy) with short breaks between RT and surgery (median ≤17 days, n = 53 patients, 46.9%) or with long breaks (median > 17 days, n = 60 patients, 53.1%). After RT, abdominoperineal resection of rectum or sphincter preserving surgery were performed. Seven patients (5.8%) did not continue treatment after radiotherapy (disqualification from the surgery or refusal).

### 3.2. HPV detection

For all 120 DNA samples, purity of isolated DNA was determined spectrophotometrically. The mean values of A260/280 and A260/230

**Table 2**
Clinical characteristics of 120 patients with rectal cancer.

| | N (%) |
|---|---|
| Age | |
| ≤ 61 years | 61 (50.8) |
| > 61 years | 59 (49.2) |
| Gender | |
| female | 34 (28.3) |
| male | 86 (71.7) |
| Clinical stage (AJCC) | |
| I | 30 (25.0) |
| II | 74 (61.7) |
| III | 16 (13.3) |
| Clinical tumour category | |
| T2 | 31 (25.8) |
| T3 | 79 (65.8) |
| T4 | 10 (8.3) |
| Clinical node category | |
| N0 | 104 (86.7) |
| N1 | 16 (13.3) |
| Grade | |
| 1 | 32 (26.7) |
| 2 | 85 (70.8) |
| 3 | 3 (2.5) |

**Table 3**
Characteristics of patients with HPV positive rectal tumours.

| Feature | Sample code | | |
|---|---|---|---|
| | 1278 | 1401 | 1456 |
| Clinical and histopathological features | | | |
| Age | 52 | 56 | 54 |
| Gender | M | M | F |
| Clinical stage | 1 | 1 | 2 |
| Grade | 2 | 2 | 2 |
| DNA extraction | | | |
| Total DNA concentration ± SE[μg] | 5.43 ± 0.61 | 6.15 ± 0.43 | 4.30 ± 0.32 |
| A260/280 | 1.74 ± 0.10 | 1.72 ± 0.12 | 1.91 ± 0.11 |
| A260/230 | 1.94 ± 0.10 | 1.62 ± 0.10 | 1.99 ± 0.14 |
| Amplification of β-actin Ct ± SE | 29.8 ± 0.4 | 27.8 ± 0.8 | 29.2 ± 0.1 |
| HPV status | | | |
| Nested PCR | negative | positive | positive |
| qPCR with HPV16 specific primers Ct ± SE | 38.42 ± 0.13 | 0.00 | 35.580 ± 0.32 |
| Amoy test | HPV16 | HPV18 | HPV16 |
| P16 immunostaining | negative | negative | negative |

Abbreviations: M - male, F – female, RT – radiotherapy, PCR – polymerase chain reaction, qPCR – quantitative polymerase chain reaction.

were 1.82 ± 0.13 (SE) and 2.05 ± 0.45, respectively. For all DNA samples, amplification of house-keeping gene of *β-actin* was noticed, with mean Ct value of 28.8 ± 0.3 (range: 23.7–38.5). Among 120 tumours, HPV positivity was detected in 2 cases (1.67%) by nested PCR (Table 3). In qPCR with specific primers for HPV16, amplification curve was also seen for 2 samples (1.67%), with the mean Ct value of 37.99 ± 1.83, however, only one of them was HPV-positive in both tests. For sample no. 1278 (positive in qPCR but negative in nested PCR), the qPCR Ct was very high (38.42), which proves very low number of HPV16 copies, probably not possible to detect by nested PCR. All three samples with HPV positivity in nested PCR and/or qPCR with specific primers for HPV16 were subjected to genotyping analysis with Amoy kit. This analysis confirmed HPV16 positivity in two cancers with viral presence in qPCR with specific primers for virus type (among them one tumour was HPV-positive in nested PCR). For one sample, which was HPV-positive in nested PCR but negative in qPCR with primers for HPV16, Amoy test revealed the presence of HPV18 infection. Clinical features of three patients with positivity of HPV DNA are presented in Table 3. All three tumours positive for DNA of HPV were negative for P16 immunostaining.

## 4. Discussion

In the present study, in the group of 120 patients with adenocarcinomas of the rectum from South–Central Poland, the percentage of HPV infection detected on the basis of PCR methods (nested PCR or qPCR) was low (1.67%) (Table 3). Moreover, all three tumours, positive for DNA of HPV, were negative for P16 immunostaining, what indicates that the presence of viral DNA may be the result of sample contamination with the content of the intestine or originate from the upper parts of the aerodigestive tract. Thus, the results of the present study suggest that infection of HPV is not related with the carcionogenesis in the rectum. However, in Poland the results concerning HPV infection in this type of tumours are conflicting (Table 1). The results presented by us are similar to those obtained by Snietura et al. [4] and Karpinski et al. [5], who did not obtain positive HPV signal in PCR in the groups of, respectively, 40 patients with large intestine adenocarcinomas from Silesia region (South-Central Poland) and 186 patients with sporadic colorectal cancers from around Wrocław (South-West Poland). Contrary, Jarzyński et al. [15] have found HPV16/18 infection in 20% of rectal tumours among 50 patients with colorectal cancer from Lublin region (eastern Poland). In turn, Miąsko et al. [16] in Podlasie region (north-eastern Poland), have shown HPV positivity in the range of 40.0% (PCR) – 47.5% (ISH HPV). In the worldwide literature, similar discrepancies are noticed (for review, see [2,13,14]). The results of some authors negate the importance of HPV infection in carcinogenesis of rectal tumours (HPV frequency ranging from 0 [3–7] to 2.8% [23]), while others indicate the presence of the virus in a meaningful percentage (from 16.0% [24] up to 84.2% [12]). On the basis of the results of their meta-analyses concerning colorectal adenomas and adenocarcinomas, Baadrup et al. [13] and Damian et al. [14] list three possible reasons explaining these contrary results: (1) differences in HPV infection rate according to geographical region, (2) different sensitivity and specificity of techniques used to the analysis of HPV prevalence and (3) date of patient's recruitment to the study.

This hypothesis about differentiation of HPV prevalence according to geographical region arises from the results of two meta-analyses concerning HPV infection in colorectal cancers [13,14]. They have shown the highest overall prevalence of HPV infection in South America (45.1%), Asia (39.2%) and the Middle East (32.2%.1), whereas the lowest in Europe (1.9%), North America (3.2%) and Australia (0.0%) [13,14]. However, the hypothesis about geographical differences in HPV infection rate in rectal cancers in Poland is rather unlikely to be true, particularly in the light of the studies concerning other types of tumours in Poland (cervical cancers, head and neck cancer). For this localization no geographical differences in HPV frequency in Poland are observed [25]. On the other hand, it should be noticed that data concerning this subject are limited and, therefore, the hypothesis about geographical differences in HPV prevalence in rectal cancer in Poland requires further studies.

Second hypothesis explaining divergences regarding HPV prevalence in rectal tumours is related to sensitivity of methods used to detect the virus. In five publications from Poland concerning rectal cancers different variants of PCR method were used: PCR – line hybridization assay (InnoLiPa) (HPV infection rate –20%) [15], PCR + electrophoresis (HPV infection from 0 to 59%) [5,16,17] and, similarly to us, qPCR with type-specific primers (0% of infected colorectal tumours [4]) (Table 3). According to the studies comparing sensitivity and specificity of INNO-LiPA assay to PGMY09/11 PCR tests (Linear Assay HPV genotyping tests) [26,27], BD Onclarity HPV Assay [27], Abbot Real Time High Risk HPV assay [28] and Genera Biosystem Pap Type assay [26], there is a very good [27,28] or complete agreement [26,29] between INNO-LiPA and the above-mentioned assays. Therefore, considering discrepancies in five studies from Poland, attention must be paid on quality of DNA used as a template in qPCR, because in these studies archival FFPE [4,15,16] or frozen tissues [5,17] were used as a source of patient's DNA. In this case, three crucial factors should be discussed: (1) date of patient's recruitment to the study, (2) type of tissues used to DNA extraction and (3) methods of DNA extraction. Date of patient's recruitment to the study is a very important parameter, due to age of FFPE blocks and the possibility of DNA degradation. In some studies, higher level of DNA degradation in older FFPE than in younger samples (0.5 *vs* 3 years and 9 *vs* 12 years) was shown [30]. Unfortunately, only in one Polish paper dates of patients' recruitment into the study are given [5]. In turn, analyzing type of tissues (frozen *vs* fixed in formalin) used to DNA extraction, it should be stated that in three studies from Poland [4,15,16] and in the present one DNA was isolated from FFPE tissues (HPV infection rate ranging from 0 to 52.5%) and in two from frozen specimens (0–59% of viral infection) [5,17]. Theoretically, more reliable results should be obtained using frozen material for DNA isolation, bbecause nucleic acids are crosslinked to proteins and DNA is degraded into smaller fragments during formalin fixation. However, Odida et al. [31] have shown a proportion of overall agreement of 86.0% in HPV detection between fresh and FFPE tissues and HPV positivity was only slightly higher in fresh tissues (90.1%) than in FFPE (88.9%). Therefore, in the light of large differences in HPV detection in Poland and taking into account

that in three Polish studies [4,15,16] the same type of material was used, it seems that this factor does not affect the described discrepancies. Considering the differences in sensitivity of HPV PCR variants, attention should be also paid on DNA extraction methods, particularly on deparaffinization method. As shown by some authors, deparaffinization with xylene is not as efficient in paraffin removal as mineral oil [32] or high temperature [33]. In turn, the presence of paraffin can result in inhibition of proteinase K (enzyme commonly used in tissue lysis during DNA extraction) and can decrease the reliability of HPV detection [34,35]. The influence of paraffin in DNA sample on HPV detection was carefully discussed in our earlier publication [36]. Unfortunately, in three Polish publications [4,5,17], the authors did not provide data concerning DNA extraction and deparaffinization

It is also worth to noticed that in none of Polish publications, in which DNA positivity was found, authors did not check activity of this infection (on the basis of P16 immunoreactivity or mRNA expression) Therefore, summarizing it should be stated that the question about incidence of HPV infection in rectal cancer in Poland is still open. The research carried out to date reveal diverging results and the reasons of these divergences are unknown. There is no doubt that we need more studies concerning this subject with a comprehensive description of the studied groups of patients and methods used to DNA isolation and detection of the virus.

## Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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