Research article

Upregulation of anaphase promoting complex (APC) 7 as a prognostic marker for esophageal squamous cell carcinoma: A hospital based study

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ABSTRACT

Esophageal cancer is the sixth leading cause of cancer death, and esophageal squamous cell carcinoma (ESCC) is the most prevalent type worldwide, with a poor prognosis due to late diagnosis. The search for new molecular prognostic biomarkers revealed that dysregulation of anaphase promoting complex/cyclosome (APC/C) activation due to altered expression of APC molecules might lead to perturbed mitotic progression leading to malignancy. We analyzed the expression of the four different subunits of the APC/C complex—APC3, APC4, APC5 and APC7—by Real Time Polymerase Chain Reaction (RT–PCR). The findings were then correlated with clinico-pathological parameters and different lifestyle factors. Significant upregulation of APC7 (tissue and blood: N = 50; 3.72 ± 1.21 and 4.45 ± 1.18, respectively) and APC3 (tissue and blood: N = 52 and 55 and 4.50 ± 1.41 and 4.58 ± 1.06, respectively) suggests their role in uncontrolled cell proliferation. In addition to their association with increasing age, their significant association with tumor size, node stage (only APC7 (p < 0.05)), and dysphagia grade supports a potential role in tumorigenic transformation in ESCC. Furthermore, several exclusive lifestyle-associated factors play a crucial supporting role in the development of ESCC in the Northeast Indian population. Various lifestyle factors, such as the duration of smoking, tobacco and betel nut consumption, and the duration of alcohol consumption, are significantly associated with the expression of APC. Analysis based on Pearson's correlation coefficient indicated a positive correlation among the gene expression levels of APC3 (both blood and tissue), APC5 (tissue) and APC3 (tissue), APC7 (tissue) and APC3 (tissue), and APC7 (tissue) and APC3 (blood). Additionally, a positive correlation was found between APC7 expression in blood and tissue samples. However, no significant correlation was found between APC 7 expression and APC4 and APC5 expression in either blood or tissue samples.

1. Introduction

Cell cycle progression through mitosis is mediated by the spatial and temporal degradation of several cell cycle checkpoint proteins through ubiquitin-mediated 26S proteasomal degradation. Protein ubiquitination is a multistep process involving three distinct enzymatic steps [1] involving E1, E2 and E3 enzymes. E3 ubiquitin ligases are key regulators of cell cycle progression and comprise mainly two subtypes—the SCF (Skp1/Cullin/F-box) and anaphase-promoting complex/cyclosome (APC/C). APC/C is primarily involved in mitotic exit via the ubiquitin-mediated degradation of Pds1/securin and cyclin B (M-phase cyclin) to maintain negligible cdk activity during late metaphase and anaphase. However, during G1 phase, a low level of mitotic cdk activity is maintained by Cdc20-and Cdh1-mediated temporal regulation of APC/C activity [2, 3, 4].

Apcc3/cdc27, a part of the conserved canonical tetratricopeptide residue (TPR) subunits of APC/C, regulates chromosomal segregation during mitosis by controlling activity following cyclin degradation. In particular, the interaction of APC3 with some proteins inhibits substrate binding to APC/C, promoting mitotic checkpoint activity. APC3 mediates...
Table 1. Comparison of APC3, APC4, APC5 & APC7 expression (Mean ± SD) between blood and tissue samples of ESCC patients (P value <0.05 is considered as significant).

| APC3 (Tissue) | APC3 (Blood) | APC4 (Tissue) | APC4 (Blood) | APC5 (Tissue) | APC5 (Blood) | APC7 (Tissue) | APC7 (Blood) |
|---------------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|
| Mean ± SD     | Mean ± SD    | Mean ± SD     | Mean ± SD    | Mean ± SD     | Mean ± SD    | Mean ± SD     | Mean ± SD    |
| 3.53 ± 2.06   | 3.76 ± 1.85  | 3.66 ± 9.55   | 6.74 ± 3.07  | 5.10 ± 4.60   | 4.25 ± 1.36  | 2.81 ± 1.71   | 3.40 ± 1.93  |

Table 2. Association of APC3, APC4, APC5 & APC7 expression (Mean ± SD) with different clinicopathological parameters in ESCC patients.

| N   | APC2 (tissue) mRNA expression | P-value | APC3 (blood) mRNA expression | P-value | APC4 (tissue) mRNA expression | P-value | APC4 (blood) mRNA expression | P-value | APC5 (tissue) mRNA expression | P-value | APC5 (blood) mRNA expression | P-value | APC7 (tissue) mRNA expression | P-value |
|-----|------------------------------|---------|------------------------------|---------|------------------------------|---------|------------------------------|---------|------------------------------|---------|------------------------------|---------|------------------------------|---------|
|     | Duration of age              |         |                              |         |                              |         |                              |         |                              |         |                              |         |                              |         |
| 30-39 years | 1.71 ± 0.74 | 0.037* | 3.16 ± 3.33 | 0.010* | 7.93 ± 4.72 | 0.831 | 3.52 ± 3.17 | 0.586 | 9.16 ± 2.17 | 0.023* | 1.31 ± 0.69 | 0.025* | 1.69 ± 0.72 |
| 40-49 years | 3.13 ± 1.22 | 0.816 | 0.99 ± 0.73 | 1.413 | 2.44 ± 3.93 | 0.417 | 5.44 ± 2.49 | 0.482 | 4.05 ± 1.81 | 0.884 | 1.86 ± 1.19 | 0.483 | 2.24 ± 1.52 |
| 50-59 years | 3.11 ± 1.55 | 0.347 | 15.09 ± 13.93 | 0.528 | 18.73 ± 13.59 | 0.347 | 4.64 ± 4.50 | 0.347 | 3.49 ± 2.25 | 0.347 | 3.03 ± 1.48 | 0.347 | 3.57 ± 1.62 |
| 60-69 years | 4.22 ± 1.13 | 0.347 | 2.39 ± 3.45 | 0.347 | 7.06 ± 4.17 | 0.347 | 7.07 ± 7.08 | 0.347 | 3.20 ± 1.84 | 0.347 | 3.07 ± 1.85 | 0.347 | 3.69 ± 2.18 |
| 70-79 years | 4.53 ± 2.55 | 0.347 | 4.67 ± 1.02 | 0.347 | 4.33 ± 9.69 | 0.347 | 3.91 ± 1.63 | 0.347 | 4.75 ± 1.16 | 0.347 | 3.61 ± 2.13 | 0.347 | 4.29 ± 2.64 |

(continued on next page)
Table 2 (continued)

| N   | APC/C (Blood) | APC/C (Lung) | APC/C (Lung) | APC/C (Lung) | APC/C (Lung) |
|-----|---------------|--------------|--------------|--------------|--------------|
|     | mRNA expression | P-value | mRNA expression | P-value | mRNA expression | P-value | mRNA expression | P-value | mRNA expression | P-value |
|     |              |          |              |          |              |          |              |          |              |          |
| 72  | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*  |
| 10  | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*  |
| 13  | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*  |
| 20  | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*    | 2.19 ± 1.98  | 0.016*  |

- Table 2: (continued)

the linking of Emi 1 (early mitotic inhibitor-1) to APC/C and subsequent prevention of chromosome missegregation during mitosis [5]. Thus, APC3 plays a crucial role in activating APC/C to recognize and degrade target substrates based on its preferential binding to either the Cdc20 or Cdh1 coactivator molecule at different stages of the cell cycle. During the G2/M transition, APC3 binds to Cdc20 and regulates UPS (ubiquitin proteasome system)-mediated degradation of securin and cyclin B to allow chromosome segregation. During the M/G1 transition, APC3-Cdh1 maintains low Cdk activity by degrading mitotic cyclins [2], Cdc25A [6], Skp2 [7] and Cks1. Additionally, it regulates the G1/S transition by degrading Geminin [8], Cdc6 [9] and its own Cyclin-A stabilizer E2, UbcH10, which inhibits the inactivation of APC/C-Cdh1.

Altered expression of APC3, a core component of APC/C, leads to either inadequate or incessant ubiquitination of securin and CyclinA1/2, CyclinB and Cyclin D1, altering the protein expression level [10]. This event results in either constitutive or delayed G0/G1 phase transition. Altered expression of APC3 has been reported in most cancers, including gastric colorectal cancer, thyroid cancer, testis cancer and lung adenocarcinoma, suggesting the role of APC3 in oncogenic pathway activation [11, 12]. Thus, APC3 upregulation may enhance tumorigenesis. Overexpression of APC3 leads to proliferation, tumor formation, migration and invasion [13]. Furthermore, its association with tumor size, tumor nodes (N), TNM grade and metastasis stage suggests its correlation with tumor progression and poor patient survival [14].

Apc4 and Apc5, in association with Apc1, form the APC/C platform subcomplex [15]. Crystal structure analysis of Apc4 [16] has revealed its bidomain architecture comprising a 360 amino acid residue containing seven bladed WD40 propeller domains (APC4WD40) and a helix bundle domain (APC4HBD) containing four α-helices. The APC4HBD domain is inserted between strands C and D of blade 4 within the extended lower surface of the propeller domain APC WD40, generating an L-shaped APC4 molecule. Additionally, another interaction between the two domains occurs through contact between β-strands A and B of blade 3 with APC4HBD and an intradomain insert of β-strand D from blade 5 between β-strands C and D of blade 6 within APC4WD40, leading to the formation of an edge β-strand (βE5). These extensive bottom surface cum strand surface contacts between the APC4WD40 and APC4HBD domains confer tertiary structural stability to APC4.

The APC5 crystal structure reveals an N-terminal 7 α helical subunit APC5N and Apc5TPR subunit containing seven TPR motifs per TPR superhelical turn [17]. The N-terminus of the Apc5TPR TPR superhelix is stabilized by interaction with the N-terminus of APC15, and the C-terminal 13 amino acid residues containing the APC1 cap fold back on the superhelix, enclosing a cavity within the APC5TPR C-terminus [16]. However, Apc5N and Apc5TPR interact via the involvement of an extensive APC4 interface, facilitating conformational changes in the APC/C platform required for Cdh1 binding-mediated stimulation of APC/C activity [18]. Furthermore, the secondary E2, UBE2S, binds to the APC2-Apc4 groove and accelerates the activity of UBE2C, maintaining a controlled E2-E3 cascade and substrate polyubiquitination.

Apc7 is a part of the tetratricopeptide repeat subunit (TPR) complex, which is the major constituent of the “arc lamp” of APC/C [19]. Studies have revealed that APC3 forms a complex with APC7 and is stabilized by APC16, forming an APC3/APC7/APC16 subcomplex [20]. Although APC16 plays a role in providing substrate stability and mitotic progression during mitosis, it is not involved in APC/C assembly. In vitro deletion analysis involving APC7 and APC16 reduces ubiquitination of the target substrates securin and cyclin B [21]. The literature suggests that positive expression of APC7 is a good prognostic factor in various types of cancer and has a prime role in pathogenesis in human colorectal carcinoma and AML [22, 23]. However, some cancers have downregulated APC7; therefore, evaluating the expression of APC7 in ESCC is crucial [22].

APC is involved in the cell cycle and controls various other substrates in regulating the cell cycle. The role of APC in cancer is evident; therefore, the role of APC in cancer progression must be investigated.
Therefore, we analyzed and compared the expression of selected APC/C subunits between blood and biopsy samples of esophageal squamous cell carcinoma by qualitative real-time PCR. Next, we assessed whether the data were correlated with various lifestyle factors (smoking, betel nut chewing, alcohol consumption and tobacco consumption), clinicopathological characteristics (tumor size, tumor node and metastasis, cell differentiation) and other physiological parameters, such as the age and sex of patients. Thus, this study will provide insight into APC/C dysregulation via altered expression of its different subunits and its association with various clinicopathological parameters in human esophageal squamous cell carcinoma (ESCC).

2. Methodology

2.1. Sample size and sample collection

A total of 70 ESCC patients (male and female) with equal numbers of age- and sex-matched healthy individuals were enrolled in our study after obtaining informed consent. The sample size was determined as previously described in Suresh et al. 2012 [24].

The diagnostic procedure included upper gastrointestinal endoscopy and pathological evaluation of tumor biopsy samples. The tumor samples and adjacent samples were obtained via biopsy from all the study participants. Standard venepuncture was used to collect blood samples. All the esophageal cancer patient samples were collected from Gauhati Medical College and Hospital, Guwahati and North East Cancer Hospital, Jorabat, Guwahati after obtaining approval from the Ethics Committee. The patients were followed up, and detailed information concerning food habits, physical activity, medical history, and lifestyle habits was collected. The data on lifestyle factors and clinicopathological parameters were divided into different groups.

2.2. RNA isolation and complementary DNA (cDNA) preparation

Total RNA was isolated manually from blood and homogenized tissue samples using TRIzol Reagent (Invitrogen, Inc., Carlsbad, CA). cDNA was prepared using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) and stored at -20°C.

2.3. mRNA expression analysis by real-time PCR

mRNA expression analysis of tissue samples was performed using a Roter-Gene Q Real-time PCR detection system (QIAGEN, Valencia, CA). The housekeeping gene β-actin was used as the reference gene to analyze gene expression. PCR amplification was performed using the SYBR Green method according to the manufacturer’s instructions. The comparative Ct (2⁻ΔΔCt) method was used to manually estimate the expression level of the studied genes.

2.4. Statistical analysis

The expression of the APC3, 4, 5 and 7 genes was analyzed with the different lifestyle factors and clinicopathological parameters using SPSS 18.0 software. All the data were expressed as means ± standard deviation. Independent-samples T test was performed for two levels, and multilevel samples were analyzed by one-way ANOVA. All the tests were 2-tailed and considered significant when the P value < 0.05. Kaplan–Meier survival analysis was performed using the log rank test, and univariate hazard analysis was performed using Cox’s regression model. Furthermore, Pearson correlation analysis was performed to determine the relationship between the study variables. The correlation coefficient values were between 1.0 and +1.0. A value of 1.0 indicates a perfect positive correlation. For a perfect negative correlation, the two
Table 3. Association of APC3, APC4, APC5 & APC7 expression (Mean ± SD) with different life style factor in ESCC patients.

| Life style factors | N  | APC3 (tissue) mRNA expression | P-value | APC4 (tissue) mRNA expression | P-value | APC5 (tissue) mRNA expression | P-value |
|--------------------|----|-------------------------------|---------|-------------------------------|---------|-------------------------------|---------|
| Smoking frequency   |    |                               |         |                               |         |                               |         |
| 1-5 times/day       | 15 | 4.39 ± 1.96                   | 0.054*  | 4.38 ± 1.79                   | 0.027*  | 5.05 ± 1.72                   | 0.046*  |
| 6-10 times/day      | 5  | 1.81 ± 1.78                   | 0.72    | 1.70 ± 0.66                   | 0.46    | 3.57 ± 1.43                   | 0.39    |
| 16-20 times/day     | 3  | 3.96 ± 1.91                   | 0.372   | 3.29 ± 1.75                   | 0.055   | 4.10 ± 1.13                   | 0.039   |
| Duration of smoking |    |                               |         |                               |         |                               |         |
| 1-10 years          | 5  | 4.10 ± 1.56                   | 0.151   | 4.31 ± 1.23                   | 0.718   | 5.16 ± 4.44                   | 0.040*  |
| 11-20 years         | 13 | 3.57 ± 1.56                   | 0.014*  | 3.69 ± 1.95                   | 0.027*  | 3.83 ± 2.37                   | 0.046*  |
| 21-30 years         | 10 | 4.81 ± 0.66                   | 0.63    | 4.71 ± 0.64                   | 0.83    | 4.63 ± 3.41                   | 0.17    |
| 31-40 years         | 4  | 5.32 ± 2.28                   | 0.215   | 5.85 ± 0.61                   | 0.575   | 6.16 ± 4.44                   | 0.11**  |
| Smoking category    |    |                               |         |                               |         |                               |         |
| No                 | 36 | 2.95 ± 1.26                   | 0.215   | 3.28 ± 1.00                   | 0.601   | 7.95 ± 6.61                   | 0.011*  |
| Yes                | 34 | 4.13 ± 1.65                   | 0.017*  | 4.26 ± 1.56                   | 0.375   | 5.15 ± 4.75                   | 0.372   |
| Duration of alcohol consumption |     |                               |         |                               |         |                               |         |
| No                 | 51 | 3.44 ± 2.04                   | 0.263   | 3.72 ± 1.75                   | 0.016*  | 7.13 ± 2.45                   | 0.414   |
| Yes                | 19 | 3.76 ± 2.15                   | 0.111   | 3.85 ± 2.14                   | 0.024   | 6.90 ± 4.63                   | 0.743   |
| Duration of betel nut consumption |     |                               |         |                               |         |                               |         |
| No                 | 22 | 2.61 ± 1.27                   | 0.005*  | 2.93 ± 1.16                   | 0.004*  | 4.63 ± 3.60                   | 0.005*  |
| Yes                | 48 | 3.95 ± 1.83                   | 0.109   | 4.14 ± 1.57                   | 0.002*  | 7.62 ± 6.83                   | 0.003*  |
| Tobacco consumption frequency |     |                               |         |                               |         |                               |         |
| 1-3 times/day       | 10 | 3.27 ± 2.07                   | 0.003*  | 3.80 ± 1.81                   | 0.001*  | 3.80 ± 1.18                   | 0.046   |
| 4-times/day         | 24 | 4.46 ± 1.72                   | 0.004*  | 4.69 ± 1.27                   | 0.001*  | 4.50 ± 0.15                   | 0.004*  |
| 7-times/day         | 6  | 4.83 ± 0.69                   | 0.003*  | 4.89 ± 0.50                   | 0.003*  | 2.27 ± 1.37                   | 0.046   |
| Duration of tobacco consumption |     |                               |         |                               |         |                               |         |
| No 0-10 years       | 11 | 3.77 ± 1.56                   | 0.002*  | 4.17 ± 1.46                   | 0.014*  | 2.72 ± 1.33                   | 0.001*  |
| 11-20 years         | 12 | 3.44 ± 2.17                   | 0.002*  | 4.09 ± 1.72                   | 0.001*  | 6.77 ± 5.13                   | 0.008*  |
| 21-30 years         | 9  | 4.64 ± 0.62                   | 0.003*  | 4.51 ± 0.71                   | 0.003*  | 2.83 ± 1.39                   | 0.003*  |
| 31-40 years         | 6  | 5.42 ± 1.82                   | 0.005*  | 5.69 ± 0.63                   | 0.005*  | 2.58 ± 1.03                   | 0.005*  |
| 41-40 years         | 2  | 5.83 ± 0.55                   | 0.005*  | 5.41 ± 0.65                   | 0.005*  | 1.28 ± 0.52                   | 0.0004* |
| Tobacco consumption category |     |                               |         |                               |         |                               |         |
| No                 | 30 | 2.61 ± 2.90                   | 0.005*  | 2.77 ± 1.96                   | 0.001*  | 3.12 ± 2.06                   | 0.014   |
| Yes                | 40 | 4.22 ± 1.76                   | 0.005*  | 4.50 ± 1.38                   | 0.0004* | 3.94 ± 2.72                   | 0.0005* |
| Smoking category/Tissue | Age group | APC3 | P-Value | APC4 | P-Value | APC5 | P-Value | APC7 | P-Value |
|------------------------|-----------|------|---------|------|---------|------|---------|------|---------|
| Yes Older Age group    | 2.94      | 0.96  | 1.43    | 3.59 | 3.60    | 2.24 | 7.95    | 0.62 | 1.15    |
| Younger Age group      | 3.66      | 0.27  | 2.17    | 3.38 | 0.50    | 3.28 | 6.98    | 0.88 | 1.27    |
| No Older Age group     | 3.06      | 0.51  | 1.21    | 3.15 | 0.72    | 0.28 | 6.91    | 0.69 | 0.71    |
| Younger Age group      | 3.15      | 0.62  | 1.06    | 3.06 | 0.56    | 3.06 | 6.91    | 0.69 | 0.71    |
| Smoking frequency       | APC3      | APC4 | APC5    | APC7 |
| 1-5 Times              | 0.75      | 0.66  | 0.97    | 1.31 |
| Younger Age group      | 0.87      | 0.87  | 1.21    | 1.27 |
| 6-10 Times             | 0.57      | 0.95  | 0.97    | 1.31 |
| Younger Age group      | 1.06      | 0.86  | 1.21    | 1.27 |
| 16-20 Times            | 0.21      | 0.56  | 0.97    | 1.31 |
| Younger Age group      | 0.21      | 0.56  | 0.97    | 1.31 |
| Duration of betel nut consumption | APC3 | APC4 | APC5 | APC7 |
| 10 years               | 0.18      | 0.51  | 0.97    | 1.31 |
| Younger Age group      | 0.17      | 0.51  | 0.97    | 1.31 |
| 20 years               | 0.42      | 0.44  | 0.71    | 0.69 |
| Younger Age group      | 0.65      | 0.37  | 0.51    | 0.56 |
| 40 years               | 0.24      | 0.54  | 0.71    | 0.69 |
| Younger Age group      | 0.14      | 0.48  | 0.71    | 0.69 |
| Duration of smoking    | APC3      | APC4 | APC5    | APC7 |
| 1-10 years             | 2.85      | 0.72  | 1.15    | 0.77 |
| Younger Age group      | 2.22      | 0.42  | 1.15    | 0.77 |
| 11-20 years            | 0.52      | 1.21  | 0.44    | 0.90 |
| Younger Age group      | 0.86      | 0.37  | 0.71    | 0.69 |
| 21-30 years            | 0.96      | 0.31  | 0.86    | 0.30 |
| Younger Age group      | 0.96      | 0.31  | 0.86    | 0.30 |
| 31-40 years            | 0.22      | 0.22  | 0.14    | 0.15 |
| Younger Age group      | 0.54      | 0.44  | 0.14    | 0.15 |
| Category of alcohol consumption | APC3 | APC4 | APC5 | APC7 |
| Yes Older Age group     | 0.012     | 0.95  | 7.12    | 0.36 |
| Younger Age group      | 0.05      | 1.05  | 0.65    | 0.49 |
| No Older Age group      | 0.59      | 0.15  | 0.72    | 0.69 |
| Younger Age group      | 0.59      | 0.15  | 0.72    | 0.69 |
| Duration of Alcohol consumption | APC3 | APC4 | APC5 | APC7 |
| 10 years               | 0.22      | 0.97  | 0.73    | 0.97 |
| Younger Age group      | 0.05      | 1.05  | 0.65    | 0.49 |
| 20 years               | 0.22      | 0.97  | 0.73    | 0.97 |
| Younger Age group      | 0.05      | 1.05  | 0.65    | 0.49 |

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Table 4. Association of expression of APC3, APC4, APC5 & APC7 between relatively younger and older age group of individuals among ESCC patients.
### Table 4 (continued)

| Smoker status | Age group | APC3 | Mean exp (Tissue) | P-Value | Mean exp (Blood) | P-Value | Mean exp (Tissue) | P-Value | Mean exp (Blood) | P-Value | Mean exp (Tissue) | P-Value | Mean exp (Blood) | P-Value |
|---------------|-----------|------|-------------------|---------|------------------|---------|-------------------|---------|------------------|---------|-------------------|---------|------------------|---------|
| **Age grouping** |           |      |                   |         |                  |         |                   |         |                  |         |                   |         |                  |         |
| Yes           | Older age group | 3.26 | 1.71              | 0.010   | 3.26             | 1.71    | 0.010             | 3.26    | 1.71             | 0.010   | 3.26             | 1.71    | 0.010             | 3.26    |
| No            | Older age group | 3.36 | 1.63              | 0.069   | 3.07             | 1.62    | 0.069             | 3.07    | 1.62             | 0.069   | 3.07             | 1.62    | 0.069             | 3.07    |
| Younger age group |         | 3.62 | 2.27              | 0.133   | 3.35             | 2.14    | 0.133             | 3.35    | 2.14             | 0.133   | 3.35             | 2.14    | 0.133             | 3.35    |
| **Frequency of tobacco consumption** |           |      |                   |         |                  |         |                   |         |                  |         |                   |         |                  |         |
| 0-10 times/day | Older age group | 3.22 | 2.20              | 0.010   | 2.56             | 2.37    | 0.010             | 2.56    | 2.37             | 0.010   | 2.56             | 2.37    | 0.010             | 2.56    |
| Younger age group |         | 3.06 | 1.93              | 0.009   | 2.78             | 1.99    | 0.009             | 2.78    | 1.99             | 0.009   | 2.78             | 1.99    | 0.009             | 2.78    |
| 11-20 times/day | Older age group | 3.23 | 2.66              | 0.010   | 3.07             | 2.13    | 0.010             | 3.07    | 2.13             | 0.010   | 3.07             | 2.13    | 0.010             | 3.07    |
| Younger age group |         | 3.39 | 2.15              | 0.009   | 2.87             | 1.94    | 0.009             | 2.87    | 1.94             | 0.009   | 2.87             | 1.94    | 0.009             | 2.87    |
| 21-30 times/day | Older age group | 3.23 | 2.66              | 0.010   | 3.07             | 2.13    | 0.010             | 3.07    | 2.13             | 0.010   | 3.07             | 2.13    | 0.010             | 3.07    |
| Younger age group |         | 3.39 | 2.15              | 0.009   | 2.87             | 1.94    | 0.009             | 2.87    | 1.94             | 0.009   | 2.87             | 1.94    | 0.009             | 2.87    |
| 31-40 times/day | Older age group | 3.31 | 2.08              | 0.009   | 2.83             | 1.88    | 0.009             | 2.83    | 1.88             | 0.009   | 2.83             | 1.88    | 0.009             | 2.83    |
| Younger age group |         | 4.44 | 0.16              | 0.006   | 4.55             | 0.05    | 0.006             | 4.55    | 0.05             | 0.006   | 4.55             | 0.05    | 0.006             | 4.55    |
| 41-50 times/day | Older age group | 4.50 | 0.36              | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    | 1.52             | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    |
| Younger age group |         | 4.50 | 0.36              | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    | 1.52             | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    |
| 51-60 times/day | Older age group | 4.50 | 0.36              | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    | 1.52             | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    |
| Younger age group |         | 4.50 | 0.36              | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    | 1.52             | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    |
| 61-70 times/day | Older age group | 4.50 | 0.36              | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    | 1.52             | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    |
| Younger age group |         | 4.50 | 0.36              | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    | 1.52             | 0.006   | 5.29             | 1.52    | 0.006             | 5.29    |

Note: The table continues with similar data for various age groups and frequency categories.
variables move in opposite directions, while a zero correlation implies no linear relationship.

2.5. Ethical declarations

This study was approved by the institutional review board of the Gauhati Medical College and Hospital, Guwahati, Assam, and all procedures were performed in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration. All patients provided written informed consent.

3. Results

3.1. Expression of the APC3, APC4, APC5 and APC7 genes in ESCC

Expression analysis of APC3, APC4, APC5 and APC7 among the 70 ESCC cases exhibited overall positive expression in both blood and tissue samples (Table 1). However, a significant difference was observed in the mean expression level (mean ± SD) between the groups (i.e., tissue and blood) for the expression of APC3 and APC7 only. A higher level of significance was obtained after analysis of the expression regulation within each group for both APC3 and APC7. Of 70 samples, 52 showed APC3 gene upregulation, with a mean fold change value of 4.5 ± 1.41, and 18 showed downregulation, with a mean value of 0.72 ± 0.23 (p = 0.001 < 0.05) in the tissue samples. APC3 gene expression in blood samples was upregulated in 55 samples, with a mean fold change value of 4.58 ± 1.06, and downregulated in 15 samples, with a mean value of 0.73 ± 0.12 (p = 0.006 < 0.05). Expression analysis of the APC4 gene showed upregulation (N = 41), with a mean fold change value of 7.75 ± 4.56, and downregulation (N = 29), with a mean value of 0.41 ± 0.12, in the tissue samples. In blood samples, the APC4 gene showed upregulation in 53 samples, with a mean fold change value of 9.27 ± 6.52, and downregulation in 17 samples, with a mean value of 0.27 ± 0.06. Thus, no significant difference was found between the groups (p = 0.224 > 0.05). The APC5 gene showed upregulation (N = 62), with a mean fold change value of 5.69 ± 4.1, and downregulation (N = 8), with a mean value of 0.55 ± 0.2, in the tissue samples (p = 0.004 < 0.05). The APC5 gene showed upregulation (N = 60), with a mean fold change value of 4.92 ± 3.36, and downregulation (N = 10), with a mean value of 0.22 ± 0.12, in the bone samples (p = 0.314 > 0.05). The APC7 gene was upregulated in 50 samples, with a mean fold change value of 3.75 ± 1.21, and downregulated in 20 samples, with a mean value of 0.737 ± 0.22 (p = 0.001 < 0.05), in the tissue samples. Further expression analysis of the APC7 gene in bone samples showed upregulation in 50 samples, with a mean fold change value of 4.45 ± 1.18, and downregulation in 20 samples, with a mean value of 0.8 ± 0.15 (p = 0.001 < 0.05) (Table 2; Figure 1).

3.2. Association of the gene expression of APC3, APC4, APC5 and APC7 in blood samples and tissue samples with clinicopathological parameters in ESCC

The association of altered expression of APC3, APC4, APC5 and APC7 in tissue and blood samples with different clinicopathological parameters, such as age, sex, dysphasia, duration of dysphasia, tumor size, tumor node, metastasis, effect of therapy, and differentiation level, in ESCC cases was analyzed (Table 2).

Age-based stratification of the ESCC cases exhibited gradual upregulation of APC3 and APC7 expression in both blood and tissue samples with increasing age, in addition to APC5 expression in blood (p = 0.023 < 0.05) (Table 2). A significant mean difference was observed in APC3 expression among the different age groups for blood (p = 0.010 < 0.05) and tissue (p = 0.037 < 0.05) samples. Similarly, the expression of APC7 also exhibited a significant mean difference in both the blood (p = 0.033 < 0.05) and tissue (p = 0.025 < 0.05) of ESCC cases from different age groups. Analysis of the association of gene expression with different dysphasia types showed significant differences in the mean expression of APC3 and APC7 in blood (p = 0.001, p = 0.025 < 0.05, respectively) and tissue (p = 0.011, p = 0.029 < 0.05) samples in addition to APC4 (p = 0.038) and APC5 (p = 0.001) expression in blood samples only. Tumor size, as a clinicopathological parameter, showed a significant association with altered expression of APC3, APC5 and APC7. The mean expression difference with advancing tumor size was significant for APC3 in tissue (p = 0.007) and blood (p = 0.004). A similar association was also observed in the expression of APC5 and APC 7 in different tumor size categories (Table 2). Tumor node stage was significantly associated with the mean expression difference in APC5 in blood samples (p = 0.027). However, the mean expression difference in APC7 in blood samples exhibited weak significance (p = 0.079) with advancing tumor node stage. Analysis of metastasis revealed a significant association with the mean expression difference of APC4 in blood (p = 0.054). Furthermore, differentiation was associated with the mean difference in APC5 in blood with advancing tumor differentiation from poorly differentiated to well-differentiated categories. However, clinicopathological parameters such as sex, dysphasia duration, and both chemotherapy and radiation therapy showed no significant association with the mean expression difference in APC/C subunits.

3.3. Association of the gene expression of the APC3, APC4, APC5 and APC7 genes in blood and tissue samples and lifestyle factors in ESCC

We also analyzed the association of APC3, APC4, APC5 and APC7 gene expression in blood and tissue samples with different lifestyle factors, such as smoking frequency, duration of smoking, smoking category, duration of alcohol consumption, category of alcohol consumption, duration of betel nut consumption, category of betel nut chewing, tobacco consumption frequency, duration of tobacco consumption, and tobacco consumption category, in ESCC patients. Considering smoking as a lifestyle factor, a significant mean difference was observed in the expression of APC3 in the tissue (p = 0.016 < 0.05) and blood samples (p = 0.027) between smokers and nonsmokers. However, APC4, APC5, and APC7 showed no significant association with smoking category within the studied cases. Further analysis of the expression association among the smokers, considering the duration and frequency of smoking as a factor, exhibited a significant difference in the mean expression of APC3 and APC7 (Table 3) between the different groups within the factor.

No significant association was found between the expression of the evaluated genes and drinking alcohol; however, the duration of drinking alcohol showed a significant mean difference in the expression of APC7 in blood samples (p = 0.017 < 0.05) among drinkers with varied durations of drinking habits.

Betel nut chewing, as a lifestyle factor, exhibited a significant mean difference in the expression of APC3 and APC7 in both tissue and blood samples between chewers and nonchewers. The mean expression of APC3 in tissue samples was 3.95 ± 1.83 for chewers (N = 48) and 2.61 ± 2.27 for nonchewers (N = 22) (p = 0.011 < 0.05). APC3 expression in blood samples was 4.14 ± 1.57 for chewers and 2.93 ± 2.16 for nonchewers (p = 0.011). Similarly, the mean expression of APC7 in tissue was 3.24 ± 1.65 for chewers and 2.59 ± 2.16 for nonchewers (p = 0.004). The expression of APC4 and APC5 exhibited no significant association with this lifestyle factor. In this study, the duration of betel nut chewing showed no significant association with altered expression of the genes.

Analysis of tobacco consumption as a lifestyle factor revealed a significant difference in the expression of APC3 and APC7 in both tissue (p = 0.001 and p = 0.004, respectively) and blood (p = 0.001 and p = 0.005, respectively) samples between consumers and nonconsumers. Further detailed analysis of this factor considering the duration of tobacco consumption and its frequency also showed a highly significant association with altered expression of APC3 and APC7 in both tissue and blood samples and APC5 in blood samples only (Table 4).
Table 5. Survival analysis data of ESCC patients.

| Parameters          | Groups     | Mean estimate | 95% C.I      | P value | Parameters          | Groups     | Mean estimate | 95% C.I      | P value |
|---------------------|------------|---------------|--------------|---------|---------------------|------------|---------------|--------------|---------|
|                     |            |       Lower Bound | Upper bound  |         |                     |            |       Lower Bound | Upper bound  |         |
| Age group           | 30–39 years | 30.71          | 18.36        | 43.06   | 0.536               | Tumor Size | 28.20         | 21.13        | 35.26   | 0.023*             |
|                     | 40–49 years | 18.28          | 5.40         | 31.16   |                     | T3         | 26.10         | 20.31        | 31.88   |                     |
|                     | 50–59 years | 23.92          | 18.15        | 29.69   |                     | T4a/b      | 17.00         | 12.45        | 21.54   |                     |
|                     | 60–69 years | 22.33          | 16.51        | 28.14   |                     | N0         | 32.11         | 22.11        | 42.54   | 0.512               |
|                     | 70–79 years | 26.66          | 17.43        | 35.26   |                     | N1         | 24.06         | 16.63        | 31.49   |                     |
|                     |            |                |              |         |                     | N2         | 22.05         | 15.47        | 28.64   |                     |
|                     |            |                |              |         |                     | N3         | 23.50         | 17.57        | 29.42   |                     |
| Gender              | Male       | 25.55          | 20.79        | 30.27   | 0.306               |            |               |              |         |                     |
|                     | Female     | 21.77          | 16.39        | 27.16   |                     |            |               |              |         |                     |
| Dysphasia           | solid      | 29.31          | 18.32        | 42.32   | 0.431               |            |               |              |         |                     |
|                     | solid + liquid | 18.20       | 6.40         | 32.31   |                     |            |               |              |         |                     |
|                     | no dysphasia | 23.90        | 20.11        | 29.31   |                     |            |               |              |         |                     |
| Duration of dysphasia | 1 month   | 30.88          | 22.36        | 38.40   | 0.216               |            |               |              |         |                     |
|                     | 2 month     | 17.60          | 11.99        | 23.20   |                     |            |               |              |         |                     |
|                     | 3 month     | 24.33          | 17.60        | 30.85   |                     |            |               |              |         |                     |
|                     | 4 month     | 19.16          | 4.54         | 33.79   |                     |            |               |              |         |                     |
|                     | 5 month     | 22.83          | 10.06        | 35.59   |                     |            |               |              |         |                     |
|                     | 6 month     | 26.19          | 15.52        | 36.47   |                     |            |               |              |         |                     |
| Chemotherapy        | yes        | 20.19          | 14.90        | 25.48   | 0.066               |            |               |              |         |                     |
|                     | no         | 27.20          | 22.48        | 31.92   |                     |            |               |              |         |                     |
| Radiation Therapy   | yes        | 20.08          | 16.012       | 24.155  | 0.003*              |            |               |              |         |                     |
|                     | no         | 32.86          | 27.061       | 38.667  |                     |            |               |              |         |                     |
| Both Therapy        | yes        | 20.30          | 16.30        | 24.30   | 0.003*              |            |               |              |         |                     |
|                     | no         | 32.95          | 26.82        | 39.08   |                     |            |               |              |         |                     |
| Metastasis          | yes        | 19.58          | 14.56        | 24.60   | 0.027*              |            |               |              |         |                     |
|                     | no         | 27.69          | 22.87        | 32.51   |                     |            |               |              |         |                     |
| Alcohol category    | Non alcoholic | 21.72       | 17.69        | 25.75   | 0.040*              |            |               |              |         |                     |
|                     | Alcoholic  | 30.47          | 23.48        | 37.46   |                     |            |               |              |         |                     |
| Duration of betel nut consumption | 1–10yrs | 25.13          | 16.47        | 33.78   | 0.655               |            |               |              |         |                     |
|                     | 11–20 yrs  | 27.78          | 20.88        | 34.69   |                     |            |               |              |         |                     |
|                     | 21–30yrs   | 25.83          | 10.43        | 41.23   |                     |            |               |              |         |                     |
|                     | 31–40yrs   | 22.08          | 13.72        | 30.44   |                     |            |               |              |         |                     |
|                     | 41–50yrs   | 19.33          | 9.06         | 29.60   |                     |            |               |              |         |                     |
| Betel nut category  | non chewers | 32.72          | 26.78        | 38.67   | 0.003*              |            |               |              |         |                     |
|                     | chewers    | 20.14          | 16.10        | 24.19   |                     |            |               |              |         |                     |
| APC3 expression in tissue | High      | 20.19          | 16.42        | 23.96   | 0.001*              |            |               |              |         |                     |
|                     | Low        | 35.38          | 28.88        | 41.89   |                     |            |               |              |         |                     |
| APC3 expression in blood  | High     | 21.00          | 17.20        | 24.79   | 0.003*              |            |               |              |         |                     |
|                     | Low        | 35.46          | 28.52        | 42.41   |                     |            |               |              |         |                     |
| APC4 expression in tissue | High    | 26.59          | 21.71        | 31.47   | 0.079               |            |               |              |         |                     |
|                     | Low        | 20.35          | 15.37        | 25.34   |                     |            |               |              |         |                     |
| APC4 expression in blood | High    | 25.83          | 21.63        | 30.03   | 0.008               |            |               |              |         |                     |
|                     | Low        | 18.70          | 12.29        | 25.11   |                     |            |               |              |         |                     |
| APC5 expression in tissue | High    | 24.93          | 21.17        | 28.70   | 0.187               |            |               |              |         |                     |
|                     | Low        | 17.62          | 6.45         | 28.79   |                     |            |               |              |         |                     |
| APC5 expression in blood | High     | 24.26          | 20.37        | 28.15   | 0.827               |            |               |              |         |                     |
|                     | Low        | 23.10          | 13.42        | 32.78   |                     |            |               |              |         |                     |
| APC7 expression in tissue | High    | 19.93          | 16.12        | 23.75   | 0.001*              |            |               |              |         |                     |
|                     | Low        | 33.81          | 27.39        | 40.22   |                     |            |               |              |         |                     |
| APC7 expression in blood | High    | 19.91          | 16.05        | 23.78   | 0.001*              |            |               |              |         |                     |
|                     | Low        | 33.22          | 26.92        | 39.53   |                     |            |               |              |         |                     |

Note: *statistically significant p value < 0.05.
3.4. Association of APC3, APC4, APC5 and APC7 expression with age considering different lifestyle factors

The expression association study with different clinicopathological parameters revealed that age was significantly associated with the altered expression of APC/C subunit genes. Further stratification based on the age of individuals as younger and older age groups demonstrated a significant association of the expression of APC3 with smoking frequency (p = 0.001), alcohol consumption category (p = 0.043) and its duration (p = 0.02) in tissue samples. For APC7, no significant association was found with any of the lifestyle factors after age-based separation into younger and older groups. However, the loss of a higher degree of significance between the expression level of the genes and lifestyle factors after age-based stratification may be due to the considerably lower number of patients in the younger age group category (N = 14) than in the older age group category (N = 56) (Table 4).
3.5. Survival analysis

All 70 ESCC patients were followed up until death or up to 45 months from the date of sampling, whichever was earlier, and Kaplan–Meier survival analysis was performed with different lifestyle and clinico-pathological factors to check their role in ESCC. Among the clinico-pathological parameters, tumor stage, metastasis, chemotherapy, radiation therapy, both chemotherapy and radiation therapy and tumor differentiation category exhibited significant associations with patient survival. Furthermore, the mean survival time for patients with different tumor stages T2, T3 and T4a/T4b was 28 months, 26 months and 17 months, respectively (p = 0.023 < 0.05). The presence or absence of metastasis also demonstrated a significant association with patient survival. Patients without metastasis showed better survival, with a mean survival time of 27 months (p = 0.027 < 0.05). Additionally, the tumor differentiation level showed higher significance (p = 0.000), with the mean survival time of patients categorized as having poor, moderate and well-differentiated tumors (38 months, 26 months, and 17 months, respectively). Interestingly, survival analysis also showed that patients receiving chemotherapy, radiation therapy or both had better survival times (p = 0.066, p = 0.003, and p = 0.003, respectively) (Table 5).

Figure 4. Plot of Kaplan-Meier analysis for duration of dysphasia.

Figure 5. Plot of Kaplan-Meier analysis for Metastasis.

Considering smoking as a lifestyle factor, a significant difference was observed in the mean survival time between smokers and nonsmokers (18 months and 29 months, respectively; p = 0.004). Similarly, a
A significant difference was also observed between tobacco chewers and nonchewers (mean survival times of 20 months and 28 months, respectively; \( p = 0.050 \)). Further analysis among the tobacco chewers considering tobacco chewing duration \( (p = 0.014) \) and frequency \( (p = 0.007) \) as factors revealed a significant difference in the mean survival time \( (Table \ 5) \) among the groups. The mean survival time for betel nut chewers was 20.14 months, and that for nonchewers was 32.72 months, indicating a significant association with this factor \( (p = 0.003) \).

Further analysis of the association between the expression of APC/C genes and survival time of patients showed a significant association with the mean expression of APC3 and APC7 only in tissue and blood samples. APC3 upregulation in tissue showed a mean survival time of 20 months, and that for blood was 21 months, indicating a significant difference \( (p = 0.001 \text{ and } p = 0.003 < 0.05, \text{ respectively}) \) with APC3 downregulation. APC7 upregulation in blood and tissue samples was also associated with the survival duration of patients \( (p = 0.001 < 0.05) \). Altered expression

Figure 6. Plot of Kaplan-Meier analysis for tumor size.

Figure 7. Plot of Kaplan-Meier analysis for smoking frequency.
of APC4 and APC5 was not significantly associated with patient survival (Figures 2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23).

3.6. Cox regression analyses and hazard outcomes

According to the univariate model of Cox regression, the hazard ratio (HR) represents the ratio of the hazard outcomes corresponding to the conditions represented by 2 groups of a variable. An HR of 1 indicates no survival difference between the 2 groups, and an HR of less than or greater than 1 indicates that one group possesses better survival than the other. According to this model, the hazard (mortality) ratio for metastatic patients was 0.54 times that of nonmetastatic patients ($p = 0.033 < 0.05$). The HR obtained for patients receiving radiation therapy was 2.71 times that of patients without radiation therapy ($p = 0.005$). Patients receiving both radiation and chemotherapy also exhibited a hazard ratio of 0.60 ($p = 0.006$) compared with nonreceivers. Among the different lifestyle factors, alcoholic patients showed an HR of 2.06 times that of the nonalcoholic group ($p = 0.05$). Further duration of alcohol consumption
also exhibited a significant (p = 0.043) hazard ratio. Similarly, the hazard ratio was 0.61 for betel nut chewers and 0.75 for tobacco chewers compared with nonchewers (p = 0.006 and p = 0.053, respectively). In addition to the lifestyle and clinicopathological parameters, altered expression of APC3 and APC7 exhibited a significant hazard ratio between the groups (p < 0.05) (Table 6).

3.7. Pearson correlation analysis of APC3, APC4, APC5 & APC7 expression in blood and tissue in ESCC

Pearson correlation analysis of the association of expression for APC3, APC4, APC5 and APC7 in tissue and blood samples revealed a significant association between APC3 and APC7 expression in both tissue and blood,
considering that the correlation was significant at the 0.01 level (2-tailed). Further survival and hazard analysis data also reflected a significant association of the expression of the gene pair (APC3 and APC7) with the mean survival duration of ESCC patients (Table 5 & Table 6). Thus, with a Pearson correlation coefficient (r) value greater than 0.7 and \( p = 0.000 < 0.05 \), the gene pair exhibits a higher degree of correlation and can be considered a potential prognostic gene pair for ESCC detection (Table 7 & Table 8).

4. Discussion

The present study analyzed the hypothesized association between altered APC/C subunit expression and tumorigenesis in esophageal carcinoma cases. Semiquantitative real-time PCR-based expression analysis showed considerable positive expression of the APC/C subunit under investigation (APC3, APC4, APC5, and APC7), specifically in blood samples compared with tissue samples.
APC3/Cdc27 expression was found upregulated in most of the blood samples (N = 55), with a mean fold change value of 4.58 ± 1.06. Comparative statistical analysis of APC3 expression in blood using different clinicopathological parameters among the studied population revealed a significant correlation with the patient age, duration of dysphasia, and tumor size. Among the different lifestyle factors, the smoking category, betel nut chewing category, duration of alcohol consumption, tobacco consumption category, and consumption frequency and duration exhibited significant associations with altered expression of APC3 in blood. An almost similar pattern of association was observed for APC3 expression in tissue. Survival analysis showed a significant impact of the abovementioned different lifestyle and clinicopathological parameters on the mean survival duration of patients (Table 5). Furthermore, survival and hazard analysis of altered APC3 expression in blood showed a mean survival time of 20 months (approx.) with upregulation of expression and a hazard ratio of 0.27 (APC3 blood expression low/ high; p = 0.007 < 0.05). Thus, APC3 upregulation possesses a potentially hazardous association with ESCC in addition to significantly affecting

Figure 14. Plot of Kaplan-Meier analysis for duration of tobacco consumption.

Figure 15. Plot of Kaplan-Meier analysis for tobacco category.
patient survival ($p = 0.003$). Furthermore, a significant mean expression difference in APC3 upregulated tissue and blood samples from patients with and without metastasis also supports APC3 upregulation-mediated advancement toward tumor metastasis. Thus, APC3 upregulation may suppress its inhibitory effect on tumorigenesis in addition to its association with a poor treatment response to radiation therapy [25]. In our study, the data obtained for APC3 expression were in good concordance when analyzed in blood and tissue samples.

APC4 expression showed a significant association with dysphasia type ($p = 0.038 < 0.05$) and metastasis ($p = 0.054$) only in the blood samples of ESCC cases. Further survival analysis data also showed no significant impact on the mean survival duration of patients ($p = 0.079$ and $0.068$ for tissue and blood, respectively). Hazard ratios of 0.611 in tissue and 1.761 in blood samples were obtained that were not significantly associated with patient survival.

APC5 expression analysis showed upregulation of expression in almost equal numbers of blood ($N = 60$) and tissue ($N = 62$) samples, with mean fold change values of $4.927 \pm 3.367$ and $5.694 \pm 4.105$, respectively. Comparative statistical analysis revealed that APC5 expression in blood was positively and directly correlated with the age of the individual in addition to the tumor size, tumor node stage and degree of differentiation ($p < 0.05$). APC 5 functions with APC7. Levine, K.N.

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Figure 16. Plot of Kaplan-Meier analysis for expression of APC3 in tissue.

Figure 17. Plot of Kaplan-Meier analysis for APC3 in blood.
et al., 2004 reported in a yeast three-hybrid study that APC5 and APC7 interact with the transcription activator CBP/p300, which has histone acetyltransferase activity and mediates transcriptional activation [26]. APC7, which is a component of the tetratricopeptide repeat motif of E3 ubiquitin ligase, is generally expressed in normal tissue at a fairly constant level and interacts with cdc20 and cdh1 to regulate the cell cycle by promoting the transition from metaphase to anaphase [27]. Semi-quantitative real-time PCR-based expression analysis of APC7 showed considerable positive expression in both tissue and blood samples, with average fold changes of 2.81 ± 1.71 and 3.40 ± 1.93, respectively.

Furthermore, the findings were compared with clinicopathological parameters. With increasing age, the mean APC7 expression increased and the mean difference between the different age groups was significant. In our study, APC7 expression was significantly upregulated in both blood and tissue samples among the different age groups. While analyzing the expression pattern of APC7 among dysphasia types, significant alterations in the expression pattern were observed. Significant upregulation of APC7 expression with advancing tumor stage from T2 to T4a/T4b was also observed in both blood and tissue samples. However, a lower level of significance was observed for APC7 expression in blood.
with advancing tumor nodes from N0 to N3. These findings are predictive of APC7 expression alteration and esophageal carcinoma tumorigenesis.

Various studies have reported that lifestyle factors, such as smoking, tobacco consumption, and betel nut chewing, are associated with different types of cancer [28, 29]. The present study also analyzed various lifestyle factors to determine their association with altered APC7 expression. A significant and strong correlation was suggested between factors such as betel nut consumption, tobacco consumption category, duration of tobacco consumption and its frequency, duration of alcohol consumption and duration of smoking and APC7 expression [30]. Our study findings were also consistent because all 51 ESCC cases (72.85%) with a history of betel nut chewing showed positive APC7 expression with a mean fold change value of 3.23 ± 1.6 in blood. Furthermore, the mean fold change in APC7 expression between tobacco chewers and

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**Figure 20.** Plot of Kaplan-Meier analysis expression of APC4 in tissue.

**Figure 21.** Plot of Kaplan-Meier analysis for expression APC4 in blood.
nonchewers was $3.36 \pm 1.60$ and $2.18 \pm 1.69$, respectively, in tissue and $3.98 \pm 1.9$ and $2.64 \pm 1.95$, respectively, in blood samples. The difference in fold change obtained was significant; following stratification based on the duration and frequency of tobacco consumption, chewers showed significant upregulation of APC7 expression with increasing duration and frequency. No significant association of APC7 expression with the alcohol consumption category was found in our study considering an alcohol consumption history up to 40 years in either the blood or tissue samples of ESCC patients. However, the duration of alcohol consumption showed a significant association in blood samples only. A significant association with the duration of smoking is another lifestyle-associated risk factor for ESCC.

Survival analysis and hazard analysis data of ESCC patients showed that the survival of esophageal cancer patients was significantly related

![Figure 22. Plot of Kaplan-Meier analysis for expression APC5 in tissue.](image)

![Figure 23. Plot of Kaplan-Meier analysis for expression of APC5 in blood.](image)
### Table 6. Hazard analysis of ESCC patients.

| Parameters                  | Group1/group2 | p value | Hazard Ratio | 95% Confidence interval | Parameters                  | Group1/group2 | p value | Hazard Ratio | 95% Confidence interval |
|-----------------------------|---------------|---------|--------------|--------------------------|-----------------------------|---------------|---------|--------------|--------------------------|
|                             |               |         |              | Lower Bound | Upper Bound             |               |         |              | Lower Bound | Upper Bound           |
| Age                         |               |         |              |             |                            |               |         |              |             |                            |
| 40 yrs-49yrs/30 yrs–39 yrs  | 0.555         | 0.665   | 0.172        | 0.532            |                             |               |         |              |             |                            |
| 50yrs-59yrs/30 yrs–39 yrs  | 0.253         | 1.954   | 2.573        | 3.056            |                             |               |         |              |             |                            |
| 60yrs-69yrs/30 yrs–39 yrs  | 0.586         | 1.275   | 0.619        | 0.598            |                             |               |         |              |             |                            |
| 70yrs-79yrs/30 yrs–39 yrs  | 0.402         | 1.468   | 6.163        | 3.603            |                             |               |         |              |             |                            |
| Gender                      |               |         |              |             |                            |               |         |              |             |                            |
| Female/Male                 | 0.319         | 1.337   | 0.755        | 2.366            |                             |               |         |              |             |                            |
| Alcohol category            |               |         |              |             |                            |               |         |              |             |                            |
| Alcoholic/Non alcoholic     | 0.050*        | 2.067   | 1.00         | 4.27             |                             |               |         |              |             |                            |
| Duration of alcohol consumption |           |         |              |             |                            |               |         |              |             |                            |
| 1-10yrs/31-40yrs           |               |         |              |             |                            |               |         |              |             |                            |
| 11-20yrs/31-40yrs          | 0.043*        | 0.372   | 0.193        | 0.968            |                             |               |         |              |             |                            |
| 21-30yrs/31-40yrs          | 0.684         | 0.794   | 0.262        | 2.406            |                             |               |         |              |             |                            |
| Betel nut category          |               |         |              |             |                            |               |         |              |             |                            |
| Chewers/Non chewers         | 0.006*        | 0.610   | 0.430        | 0.867            |                             |               |         |              |             |                            |
| Duration of betel nut consumption |           |         |              |             |                            |               |         |              |             |                            |
| 1-10yrs                     | 0.328         | 3.122   | 0.756        | 2.310            |                             |               |         |              |             |                            |
| 11-20 yrs                  |               |         |              |             |                            |               |         |              |             |                            |
| 21-30yrs                  | 0.276         | 0.721   | 0.40         | 1.299            |                             |               |         |              |             |                            |
| Duration of dysphasia       |               |         |              |             |                            |               |         |              |             |                            |
| 2 month/1month              | 0.437         | 0.641   | 0.209        | 1.965            |                             |               |         |              |             |                            |
| 3 month/1month              | 0.231         | 1.869   | 0.672        | 5.20             |                             |               |         |              |             |                            |
| 4 month/1month              | 0.792         | 1.142   | 0.409        | 3.221            |                             |               |         |              |             |                            |
| Tobacco category            |               |         |              |             |                            |               |         |              |             |                            |
| Chewers/Non chewers         | 0.053         | 0.751   | 0.558        | 1.01             |                             |               |         |              |             |                            |
| Frequency of tobacco consumption |           |         |              |             |                            |               |         |              |             |                            |
| 1-3 times per day          | 0.157         | 0.701   | 0.429        | 1.14             |                             |               |         |              |             |                            |
| 4-6 times per day          | 0.063         | 0.476   | 0.218        | 1.04             |                             |               |         |              |             |                            |
| 6-10 times per day         | 0.045*        | 1.614   | 1.011        | 2.577            |                             |               |         |              |             |                            |
| Metastasis                  |               |         |              |             |                            |               |         |              |             |                            |
| Present/Absent              | 0.003*        | 0.539   | 0.305        | 0.953            |                             |               |         |              |             |                            |
| Smoking category            |               |         |              |             |                            |               |         |              |             |                            |
| Smokers/Non smokers         | 0.006*        | 0.666   | 0.499        | 0.889            |                             |               |         |              |             |                            |
| Smoking frequency           |               |         |              |             |                            |               |         |              |             |                            |
| 6-10/1-5 times per day     | 0.726         | 0.754   | 0.155        | 3.66             |                             |               |         |              |             |                            |
| 16-20/1-5 times per day    | 0.502         | 0.510   | 0.071        | 3.64             |                             |               |         |              |             |                            |
| Duration of smoking         |               |         |              |             |                            |               |         |              |             |                            |
| 1-10yrs/31-40yrs           | 0.799         | 1.198   | 0.298        | 4.80             |                             |               |         |              |             |                            |
| 11-20 yrs/31-40yrs         | 0.862         | 1.109   | 0.346        | 3.55             |                             |               |         |              |             |                            |
| 21-30yrs/31-40yrs          | 0.492         | 1.51    | 0.462        | 4.96             |                             |               |         |              |             |                            |
| Tumor size                  |               |         |              |             |                            |               |         |              |             |                            |
| T2/T 4/4b                  | 0.131         | 0.707   | 0.451        | 1.109            |                             |               |         |              |             |                            |
| T3/T 4a/4b                 | 0.368         | 0.834   | 0.561        | 1.239            |                             |               |         |              |             |                            |
| Chemotherapy                |               |         |              |             |                            |               |         |              |             |                            |
| Yes/No                      | 0.075         | 0.773   | 0.582        | 1.027            |                             |               |         |              |             |                            |
| Radiation Therapy           |               |         |              |             |                            |               |         |              |             |                            |
| Yes/No                      | 0.005         | 2.71    | 1.346        | 5.458            |                             |               |         |              |             |                            |
| Both Therapy                | 0.006         | 0.600   | 0.417        | 0.864            |                             |               |         |              |             |                            |
to differences in tumor stage, presence or absence of metastasis, tumor differentiation, habit of chewing betel nut and tobacco, smoking habit, and frequency of tobacco consumption per day. Additionally, hazard analysis data supported the effect of these parameters on the survival of esophageal cancer patients. During the present study, the hazard ratio was significant in patients with APC3 and APC7 upregulation in blood and tissue, metastasis, consuming betel nut and tobacco, prolonged duration of drinking habit from 11 to 20 years or more and frequency of tobacco consumption 10 times per day. These data represent the influence of these parameters on the survival and mortality rate of esophageal cancer patients.

The overall heightened expression of APC3 and APC7 in most of the ESCC samples contradicts the study reported by Park et al. 2005 [31]. That study showed that APC7 is downregulated in breast carcinoma.

### Table 7. Association of APC3, APC4, APC5 & APC7 expression in blood and tissue level in ESCC patients.

| Parameters                                           | p-value | Pearson correlation |
|------------------------------------------------------|---------|---------------------|
| Expression of APC3 (blood) and expression of APC3 (tissue) | 0.000*  | 0.746               |
| Expression of APC4 (tissue) and expression of APC3 (tissue) | 0.411   | 0.100               |
| Expression of APC4 (tissue) and expression of APC3 (blood) | 0.277   | 0.132               |
| Expression of APC4 (blood) and expression of APC3(tissue) | 0.680   | 0.050               |
| Expression of APC4 (blood) and expression of APC3 (blood) | 0.756   | 0.038               |
| Expression of APC4 (blood) and expression of APC4 (tissue) | 0.559   | 0.071               |
| Expression of APC5 (tissue) and expression of APC3 (tissue) | 0.020   | 0.279               |
| Expression of APC5 (tissue) and expression of APC3 (blood) | 0.832   | -0.026              |
| Expression of APC5 (tissue) and expression of APC4 (tissue) | 0.970   | -0.005              |
| Expression of APC5 (blood) and expression of APC3 (tissue) | 0.222   | -0.148              |
| Expression of APC5 (blood) and expression of APC3 (blood) | 0.239   | -0.143              |
| Expression of APC5 (blood) and expression of APC3 (tissue) | 0.192   | -0.158              |
| Expression of APC5 (blood) and expression of APC4 (tissue) | 0.542   | 0.074               |
| Expression of APC5 (blood) and expression of APC4 (blood) | 0.194   | -0.157              |
| Expression of APC5 (blood) and expression of APC5 (tissue) | 0.620   | -0.060              |
| Expression of APC7 (tissue) and expression of APC3 (tissue) | 0.000*  | 0.750               |
| Expression of APC7 (tissue) and expression of APC3 (blood) | 0.000*  | 0.726               |
| Expression of APC7 (tissue) and expression of APC4 (tissue) | 0.729   | 0.042               |
| Expression of APC7 (tissue) and expression of APC4 (blood) | 0.517   | 0.079               |
| Expression of APC7 (tissue) and expression of APC5 (tissue) | 0.648   | -0.055              |
| Expression of APC7 (tissue) and expression of APC5 (blood) | 0.138   | -0.179              |
| Expression of APC7 (blood) and expression of APC3 (tissue) | 0.000*  | 0.592               |
| Expression of APC7 (blood) and expression of APC3 (blood) | 0.000*  | 0.768               |
| Expression of APC7 (blood) and expression of APC5 (tissue) | 0.849   | -0.023              |
| Expression of APC7 (blood) and expression of APC4 (blood) | 0.643   | 0.056               |
| Expression of APC7 (blood) and expression of APC5 (blood) | 0.624   | -0.060              |
| Expression of APC7 (blood) and expression of APC5 (tissue) | 0.764   | -0.036              |
| Expression of APC7 (blood) and expression of APC7 (tissue) | 0.000*  | 0.790               |

Note: *statistically significant p value < 0.05.

### Table 8. Pearson correlation (r²) of APC3, APC4, APC5 & APC7 expression in blood and tissue in ESCC patients.

|         | APC3 (tissue) | APC3 (blood) | APC4 (tissue) | APC4 (blood) | APC5 (tissue) | APC5 (blood) | APC7 (tissue) | APC7 (blood) |
|---------|---------------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|
| APC3 (tissue) | r² 1          | 0.556        | 0.010         | 0.003        | 0.014         | 0.020        | 0.562         | 0.350        |
|         | p-value       | 0.000*       | 0.411         | 0.680        | 0.329         | 0.239        | 0.000*        | 0.000*       |
| APC3 (blood) | r² 0.556      | 1            | 0.017         | 0.001        | 0.001         | 0.024        | 0.527         | 0.589        |
|         | p-value       | 0.000*       | 0.277         | 0.756        | 0.832         | 0.192        | 0.000*        | 0.000*       |
| APC4 (tissue) | r² 0.010      | 0.017        | 1             | 0.005        | 0.000         | 0.005        | 0.002         | 0.001        |
|         | p-value       | 0.411        | 0.277         | 0.559        | 0.970         | 0.542        | 0.729         | 0.849        |
| APC4 (blood) | r² 0.003      | 0.001        | 0.005         | 1            | 0.021         | 0.024        | 0.006         | 0.003        |
|         | p-value       | 0.680        | 0.756         | 0.559        | 0.222         | 0.194        | 0.517         | 0.643        |
| APC5 (tissue) | r² 0.013      | 0.001        | 0.000         | 0.021        | 1             | 0.004        | 0.003         | 0.004        |
|         | p-value       | 0.329        | 0.832         | 0.970        | 0.222         | 0.620        | 0.648         | 0.624        |
| APC5 (blood) | r² 0.020      | 0.024        | 0.005         | 0.024        | 0.004         | 1            | 0.003         | 0.001        |
|         | p-value       | 0.239        | 0.192         | 0.542        | 0.194         | 0.620        | 0.138         | 0.764        |
| APC7 (tissue) | r² 0.562      | 0.527        | 0.002         | 0.006        | 0.003         | 0.032        | 1             | 0.624        |
|         | p-value       | 0.000*       | 0.000*        | 0.729        | 0.517         | 0.648        | 0.138         | 0.000*       |
| APC7 (blood) | r² 0.350      | 0.589        | 0.001         | 0.003        | 0.004         | 0.001        | 0.624         | 1            |
|         | p-value       | 0.000*       | 0.000*        | 0.849        | 0.643         | 0.624        | 0.764         | 0.000*       |

Note: *statistically significant p value < 0.05.
accompanied by the loss of another subunit, APC3, suggesting heterogeneous regulation of APC components in breast carcinomas. However, our data are consistent with other studies showing that the expression patterns of APC components are not simultaneously modulated [32] and that the components of APC can be individually modulated by environmental stimuli [30]. Differential modulation of APC subunits and dysregulation of APC activation can induce unscheduled mitotic progression of malignant stimuli [30]. Differential modulation of APC subunits and dysregulation of APC activation can induce unscheduled mitotic progression of malignant stimuli [30].

Our two-tailed correlation analysis of APC/C gene expression in blood and tumor tissue samples in ESCC patients demonstrated a more common expression pattern of APC3, APC5 and APC7 in blood and tissue samples from ESCC patients. Thus, the APC3, APC5 and APC7 expression levels in blood may be prognostic markers.

Different clinicopathological and molecular parameters, such as tumor size, tumor node, degree of differentiation, lymph node metastasis, histopathology grade, lymphovascular invasion, and alteration of oncogene expression, were reported to be good prognostic markers for various types of cancer detection [34]. In the present study, strong prognostic indicators of tumorigenesis, such as a high histopathologic grade, a high rate of proliferation, tumor node stage and probable degree of differentiation, were associated with positive expression of APC3, APC5 and APC7 in ESCC patients, suggesting poor survival. Furthermore, more significant APC3 and APC7 positive expression in blood may support that positive expression of the APC3 and APC7 gene pair is a viable prognostic marker for a proper ESCC prognosis. More in-depth molecular epidemiological studies will help elucidate the role of the APC3 and APC7 genes and their interaction with the different lifestyle factors and clinicopathological relationships, contributing to the development of ESCC in Northeast India.

Declarations

**Author contribution statement**

Eyashin Ali: Conceived and designed the experiments.
Manash Jyoti Kalita: Analyzed and interpreted the data; Wrote the paper.
Simanta Kalita and Jasmin Sultana: Wrote the paper.
Jayasree Talukdar: Performed the experiments; Analyzed and interpreted the data.
Ankur Jyoti Deka, Md. Ghaznavi Idris and Sahana Bhattacharjee: Analyzed and interpreted the data.
Bikash Narayan Choudhury and Munindra Narayan Baruah: Analyzed and interpreted the data.
Subhash Medhi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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**Data availability statement**

Data included in article supplementary material/referenced in article.

**Declaration of interests statement**

The authors declare no conflict of interest.

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**Additional information**

No additional information is available for this paper.

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