A novel cancer testis antigen target A-kinase anchor protein (AKAP4) for the early diagnosis and immunotherapy of colon cancer

Nirmala Jagadish, Deepak Parashar, Namita Gupta, Sumit Agarwal, Aditi Sharma, Rukhsar Fatima, Vaishali Suri, Rajive Kumar, Anju Gupta, Nirmal Kumar Lohiya and Anil Suri

ABSTRACT

Colorectal cancer (CRC) is mainly a disease of developed countries and a major cause of death worldwide. The present study was undertaken to investigate the association of novel cancer testis (CT) antigen, A-kinase anchor protein (AKAP4) with CRC. AKAP4 gene and protein was examined by RT-PCR, in situ hybridization and immunohistochemistry (IHC) in 200 clinical specimens of different stages and grades. In addition, humoral response against AKAP4 was detected by enzyme-linked immunosorbent assay and Western blotting in 172 available sera samples of CRC patients. We observed that majority of CRC patients demonstrated AKAP4 expression and elicited immune response. AKAP4 protein expression, based on immunoreactivity score (IRS) predicted presence of CRC with 84% sensitivity, 100% specificity, 100% of positive predictive value (PPV) and 83.33% negative predictive value (NPV). Humoral response against AKAP4 protein was generated in 82% of the CRC patients. Further, statistical analysis revealed that antibodies found against AKAP4 in CRC patients predicted presence of malignancy with 81.98% sensitivity, 100% specificity, 100% PPV, and 63.53% NPV. Collectively, our data suggests that the majority of CRC cases demonstrate the association of AKAP4 in CRC. AKAP4 gene and protein was examined by RT-PCR, in situ hybridization and immunohistochemistry (IHC) in 200 clinical specimens of different stages and grades. AKAP4 expression in cancerous tissues and its potent immunogenicity, AKAP4 is considered as a promising biomarker and immunotherapeutic candidate. AKAPs are specialized anchoring proteins that recruit and compartmentalize protein kinase A (PKA) and other enzymes in the cytoplasm to specific sub-cellular locations and organelles for their enzymatic functions. AKAP4, being a scaffolding protein, anchors cAMP- dependent PKA and modulates the downstream signaling module. PKA has been proposed to be involved in majority of human tumors and malignant properties including cell proliferation, angiogenesis, and chemo-resistance. This usefulness of AKAP4 as a biomarker and its potential as a candidate molecule for immunotherapy has been demonstrated in breast, EOC, cervix cancer. However, the role of AKAP4 has not been studied in CRC so far. To the best of our knowledge, ours is the first study demonstrating the association of AKAP4 in CRC.

In the present study, we investigated the expression of AKAP4 gene and protein in various clinical stages and histopathological grades of CRC tumors and evaluated the humoral response in these patients. Our data suggests that AKAP4 is expressed in early stages and thus, could be used as a biomarker for early diagnosis of CRC. Further, it may be a promising target for immunotherapy in CRC management.
Results

**AKAP4 gene is expressed in majority of CRC specimens**

RT-PCR was carried using AKAP4 specific primers, to detect the AKAP4 transcript in CRC and ANCT specimens (Fig. 1). Our results showed that 84% of CRC specimens were found positive for AKAP4 transcript (Table 1). However, no AKAP4 mRNA was detected in ANCT specimens. We further analyzed our data that revealed AKAP4 transcript was found in 88% (7/8) stage I, 84% (31/37) stage II, 88% (92/104) stage III, and 74% (38/51) stage IV patients whereas no AKAP4 gene expression was detected in ANCT specimens (Table 1). Based on the histological classification, 88% (63/71) of well differentiated, 83% (85/102) of moderately differentiated and 74% (20/27) poorly differentiated specimens showed AKAP4 gene expression (Table 1). Of the 155 specimens found positive for lymph node involvement, 84% (130/155) specimens expressed AKAP4 gene while 84% (38/45) of specimens negative for lymph node involvement showed AKAP4 expression. Similarly in the presence or absence of metastasis, 74% (38/51) of patients found positive with metastasis expressed AKAP4 gene while, 87% (130/149) of patients negative for metastasis expressed AKAP4 gene. Testis cDNA was also subjected for RT-PCR as a positive control.

![Figure 1. AKAP4 gene expression in CRC patient specimens. RT-PCR analysis shows AKAP4 transcript in representative specimens of stage I, II, III, and IV, WD, MD, PD grades of CRC, whereas, ANCT specimens failed to express AKAP4 mRNA. Testis mRNA was used as positive control and β-actin as loading control. (WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated).](image)

| Table 1. Clinicopathological characteristics of colorectal carcinoma patients: AKAP4 expression and humoral response. |
|-------------------------------------------------------------|
|                  | IHC (%) of tissue | RT-PCR (%) of tissue | ELISA (%) of serum |
| All tumors       | 168/200 (84)      | 168/200 (84)         | 141/172 (82)       |
| Adjacent normal cancerous tissues | 0/160 (0%) | 0/160 (0%) | - |
| Tumor stages     |                  |                      |                  |
| Stage I          | 7/8 (88)         | 7/8 (88)             | 7/8 (87)          |
| Stage II         | 31/37 (84)       | 31/37 (84)           | 32/37 (86)        |
| Early stages (I + II) | 38/45 (84) | 38/45 (84) | 39/45 (86) |
| Stage III        | 92/104 (88)      | 92/104 (88)          | 63/76 (82)        |
| Stage IV         | 38/51 (74)       | 38/51 (74)           | 39/51 (76)        |
| Late stages (III + IV) | 130/155 (84) | 130/155 (84) | 102/127 (80) |
| Histological grades |                  |                      |                  |
| Well differentiated | 63/71 (88)      | 63/71 (88)           | 41/49 (83)       |
| Moderately differentiated | 85/102 (83)     | 85/102 (83)          | 79/96 (82)       |
| Poorly differentiated | 20/27 (74)       | 20/27 (74)           | 21/27 (77)       |
| Lymph node involvement |                |                      |                  |
| Positive         | 130/155 (84)     | 130/155 (84)         | 102/127 (80)     |
| Negative         | 38/45 (84)       | 38/45 (84)           | 39/45 (86)       |
| Metastasis       |                  |                      |                  |
| Positive         | 38/51 (74)       | 38/51 (74)           | 39/51 (76)       |
| Negative         | 130/149 (87)     | 130/149 (87)         | 102/121 (84)     |

Statistical analysis (p values of different test used in this study)

|                  | Mann-Whitney U-test (p-value) | Pearson’s χ2 test (p-value) | Kruskal–Wallis Test |
|------------------|-------------------------------|-----------------------------|---------------------|
|                  | RT-PCR/IHC | ELISA | RT-PCR/IHC | ELISA | RT-PCR/IHC | ELISA |
| Tumor stage I + II | 0.624     | 0.003† | 0.793 | 0.939 | - | - |
| Tumor stage II + III | 0.036†    | 0.014† | 0.464 | 0.624 | - | - |
| Tumor stage III + IV | 0.028†   | 0.001† | 0.026 | 0.372 | - | - |
| Tumor stage I, II, and IIIUV | 0.052 | 0.015† | 0.926 | 0.341 | - | - |
| Grades WD + MD     | 0.209 | 0.084 | 0.320 | 0.835 | - | - |
| Grades WD + PD     | 0.036† | 0.006† | 0.072 | 0.526 | - | - |
| Grades MD + PD     | 0.290 | 0.456 | 0.272 | 0.595 | - | - |
| Grades WD, MD + PD | - | - | 0.119 | - | - | - |
| Lymph node positivity | 0.053 | 0.018† | 0.926 | 0.341 | - | - |
| Metastasis positivity | 0.101 | 0.28 | 0.032† | 0.223 | - | - |

ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; MD, moderately differentiated; WD, well differentiated.

†p < 0.05, statistically significant.

Statistical analysis (p values of different test used in this study).
Expression of AKAP4 gene and protein is cell type specific

In order to detect cell type AKAP4 gene and protein expression, serial CRC specimen sections were subjected to in situ RNA hybridization studies employing in-vitro synthesized anti-sense and sense AKAP4 riboprobes and by IHC. AKAP4 gene expression was confirmed using anti-sense riboprobe in 88% (7/8) stage I, 84% (31/37) stage II, 88% (92/104) stage III, and 74% (38/51) stage IV patients (Fig. 2 and Table 1). However, ANCT specimen sections did not reveal hybridization with anti-sense riboprobes (Table 1). CRC specimen classified as per TNM also confirmed that of the 155 specimens found positive for lymph node involvement, 84% (130) specimens expressed AKAP4 gene while 84% (38/45) of specimens negative for lymph node involvement showed AKAP4 expression. In addition, based on the histopathological grades, 88% (63/71) well differentiated, 83% (85/102) moderately differentiated and 74% (20/27) poorly differentiated specimens revealed AKAP4 gene expression. However, as expected sense riboprobe failed to hybridize with AKAP4 transcript in CRC specimens (Fig. 2).

Further, AKAP4 protein expression was validated by IHC in serial CRC specimen sections which showed 84% of CRC patients expressed endogenous AKAP4 protein. Our analysis revealed that 88% (7/8) stage I, 84% (31/37) stage II, 88% (92/104) stage III, and 74% (38/51) stage IV patients expressed AKAP4 protein expression, whereas no AKAP4 protein expression was detected in ANCT specimens (Fig. 3). Based on the histopathological grades, 88% (63/71) well differentiated, 83% (85/102) moderately differentiated and 74% (20/27) poorly differentiated specimens showed AKAP4 protein expression (Table 1). Moreover, specimens found positive for lymph nodes involvement, 84% (130) specimens expressed AKAP4 protein while 84% (38/45) of specimens negative for lymph node involvement showed AKAP4 protein expression.

We examined our data based on immuno-reactivity score (IRS) which is depicted in Fig. 4A. Statistical analysis using Mann-Whitney U-test revealed significant difference in AKAP4 protein expression in stage II (IRS = 59.19 ± 2.38) and III (IRS = 64.15 ± 0.32, p = 0.036) and stage III (IRS = 64.15 ± 0.32,) and IV (IRS = 56.03 ± 3.50, p = 0.028) respectively. Similarly, well differentiated (IRS = 63.54 ± 1.91) and poorly differentiated (IRS = 55.90 ± 3.22) specimens of CRC patients also showed significant difference for AKAP4 protein expression (p = 0.036). Interestingly, significant (p = 0.026) association was found between stage III (IRS = 64.15 ± 0.32) and IV (IRS = 56.03 ± 3.50) for AKAP4 protein expression.

---

**Figure 2.** Cell type specific AKAP4 gene expression by RNA in situ hybridization. Left panel shows the representative micrographs of Haemotoxylin and Eosin (H&E) staining in stage I, II, III, and IV CRC patients. Middle panel shows the presence of AKAP4 gene as depicted by violet blue reactivity probed with anti-sense riboprobes. Right panel shows no hybridization of AKAP4 gene in CRC patient specimens probed with sense riboprobes. Original magnification 200×, objective 20×.
using Pearson’s $\chi^2$ test. Significant association of AKAP4 protein expression was also observed between the patients with presence (IRS = 56.03 ± 3.50) and absence (IRS = 62.63 ± 1.31) of metastasis ($p = 0.032$). Kruskal–Wallis test demonstrated the significant difference ($p = 0.024$) in AKAP4 gene and protein expression among different stages I (IRS = 57.86 ± 1.40), II (IRS = 59.19 ± 2.38); III (IRS = 64.15 ± 0.32); and IV (IRS = 56.03 ± 3.50) of CRC. Moreover, AKAP4 protein expression based on IRS predicted presence of CRC with 84% sensitivity, 100% specificity, 100% of PPV, and 83.33% NPV.

We further analyzed our data based on IRS and divided the CRC specimens in two groups as depicted in Fig 4B. Group I included CRC specimens which showed >50% cells expressing AKAP4 protein and Group II included CRC specimens which showed <50% cells expressing AKAP4 protein. It was interesting to note that higher number (76.79%; 129 of 168) of CRC patients expressed AKAP4 protein in Group I (IRS = 68.44 ± 0.92) as compared to Group II CRC patients (23.21%; 39 of 168, IRS = 36.97 ± 1.55). Similarly, among the grades higher IRS was observed in well differentiated (IRS = 63.54 ± 1.91; 63 of 168) as compared to moderately differentiated (IRS = 60.59 ± 1.98, 85 of 168) and poorly differentiated (IRS = 55.90 ± 3.22, 20 of 168) histopathological characteristics. However, when we compared early stages (stage I and stage II) and late stages (stage III and stage IV) IRS, we did not observe significant difference ($p = 0.052$) using Mann-Whitney U-test (Table 1).

**Humoral response**

As we observed AKAP4 protein expression in various stages and histopathological grades of CRC specimens, we further investigated the humoral response against AKAP4 in the sera of available 172 CRC patients. Initially, using healthy donor sera ($n = 138$), a cut-off value was calculated based on mean value of absorbance plus two times of standard deviation [SD, (mean + 2SD) $0.164 + 2 \times 0.054 = 0.272$]. The values detected in patients’ sera above cut-off value of 0.272 were considered positive for anti-AKAP4 antibodies (Fig. 5). Our results revealed, 82% (141/172) patients showed humoral response against AKAP4 (Table 1). Among various stages 87% (78/87) of stage I, 86% (32/37) of stage II, 82% (63/76) of stage III, and 76% (39/51) of stage IV patients generated circulating anti-AKAP4 antibodies. Similarly, 83% (41/49) well differentiated, 82% (79/96) moderately differentiated, and 77% (21/27) poorly differentiated CRC patients demonstrated the presence of circulating anti-AKAP4 antibodies. In addition, 80% (102/127)

![Figure 3. IHC analyses of AKP4 protein expression in CRC patients. First panel shows representative images of the H&E stained tissue specimens of stage I, II, III, and IV patients. Second panel shows the immunoreactivity against AKAP4 protein indicating cytoplasmic localization (brown color) in serial tissue section probed with anti-AKAP4 antibody. Third panel shows no reactivity in tissue sections probed with control IgG antibody. Fourth panel depicts ANCT specimens which fail to show any immuno-reactivity probed with anti-AKAP4 antibody. Bottom panel shows the IHC images of normal colon tissue which failed to show immuno-staining probed with anti-AKAP4 antibody. Original magnification 200x, objective 20x.](image-url)
patient’s positive for lymph node involvement, while, 86% (39/45) patients negative for lymph node involvement were found to have humoral response against AKAP4. Patients with the presence of metastasis exhibited humoral response against AKAP4 in 76% (39/51), while, 84% (102/121) patients found negative for metastasis generated humoral response against AKAP4 (Table 1).

It was interesting to note that using Mann-Whitney U-test, significant difference was found in humoral response generated against AKAP4 between sera of stage I and II patients (p = 0.003), stage II and III patients (p = 0.014), and stage III and IV (p = 0.001) patients (Table 1). In addition, the significant difference (p = 0.015) was also seen between early (I and II) and late stage (III and IV) CRC patients (Table 1). Similarly, a significant difference (p = 0.006) was observed between well differentiated and poorly differentiated type sera samples of CRC patients (Table 1). The patients with presence and absence of lymph node in CRC patients also demonstrated significant difference (p = 0.018) (Table 1). Using Kruskal–Wallis test it was observed that significant difference existed among various stages (I, II, III, and IV, p = 0.000) and different histotypes of CRC (p = 0.046) (Table 1). Further, statistical analysis revealed that antibodies found against AKAP4 in CRC patients predicted presence of malignancy with 81.98% sensitivity, 100% specificity, 100% PPV, and 63.53% NPV.

Subsequently, to validate the presence of anti-AKAP4 antibodies in patients sera, Western blotting was carried out (Fig. 5). Sera of all stages and grades of CRC patients found positive for circulating anti-AKAP4 antibodies by ELISA showed distinct immuno-reactivity against AKAP4 protein in Western blotting. No immuno-reactivity was detected in the sera of the healthy donors or found negative for anti-AKAP4 antibodies in CRC patients in ELISA. Furthermore, the specific humoral response against AKAP4 protein was carried out in patient’s sera by neutralization experiments which showed complete loss of immuno-reactivity with AKAP4 protein (Fig. 5). Thus, the humoral response against AKAP4 indicates its potential as a biomarker for early detection and diagnosis for CRC.

**Discussion**

CRC is one of the leading causes of cancer-related mortality throughout the world.1 Most often geographical variations are observed in CRC occurrence in developed and developing countries.7 Hence, only CRC screening tests allow the detection and removal of CRC polyps before they progress to cancer. The standard treatment modality for CRC is tumor resection through surgery which is possible at early stages when metastasis has not begun. However, the detection of CRC at early stages is difficult and moreover, early stage cancer does not have symptoms that can lead to the cancer. Thus, there is a need to identify and characterize a candidate molecule that may be associated with CRC during early stages.

Recently, CT antigens have been found to be associated with various malignancies and have been proposed to be an ideal target as biomarker for early detection and immunotherapy.4,19 In this context, few CT antigens gene expression has been validated in CRC specimens.3,6 For example, OY-TES-1 protein was detected only in 43.3% (26/60) CRC patients.7 While, NY-ESO-1 and MAGE-A3 protein expression was found to be upto 10% and 8% respectively in CRC patients.8,9 Interestingly, none of these studies reported humoral response against CT antigens except OY-TES-1 which demonstrated that only 9.6% (7/73) patients generated auto-antibodies.7 The most studied SPAG9 in various malignancies20–25 was also shown to be associated with CRC in various stages and histotypes.8 SPAG9 was also found to be highly immunogenic and demonstrated humoral response in CRC patients.6 At present, none of these molecules have been included in clinical practice for CRC management. Therefore, in the present investigation we assessed a novel CT antigen AKAP4 gene and protein expression and humoral response in various stages and clinicopathological CRC patient specimens. This may contribute in developing a serum-based biomarker for early detection and diagnosis of CRC.

AKAP4 is novel protein which is only expressed in testis and not any other somatic tissues.11 To the best of knowledge, ours is a first study reporting an association of AKAP4 with CRC. Interestingly, based on quantitative analysis by counting the AKAP4 immuno-reactive positive cells, we observed significant difference (p < 0.001) between ANCT and malignant CRC.
specimen tissue sections. Further, our data indicated that majority of CRC patients (84%, 168 of 200) demonstrated AKAP4 expression irrespective of stages and grades suggesting a possible role of AKAP4 in CRC. We have shown earlier that AKAP4 has been documented to be upregulated in breast cancer patients (85%),\textsuperscript{12} epithelial ovarian carcinoma (EOC) patients (89%),\textsuperscript{13} and cervix cancer patients (86%).\textsuperscript{14} Moreover, other laboratories have also confirmed AKAP4 protein expression in 65% prostate cancer patients,\textsuperscript{16} 25% NSCLC patients,\textsuperscript{17} and 42% multiple myeloma patients.\textsuperscript{15} Thus, AKAP4 expression in CRC patients may be a potential target molecule for immunotherapy.

Figure 5. Humoral response against AKAP4 protein. (A) Sera from CRC patients were analyzed by ELISA to examine humoral response against AKAP4 protein. Line X denotes the cut-off value (mean ± 2SD; X = 0.272) obtained from the healthy donors sera. The patient’s sera value above line X denotes positive for anti-AKAP4 antibodies while those below line X were considered to be negative for anti-AKAP4 antibodies. (B) Western blotting of the sera obtained from CRC patients. M: marker, Lane 1 shows coomassie stained recombinant AKAP4 protein. Lane 2 shows recombinant AKAP4 protein probed with anti-AKAP4 antibody. Lane 3 shows no immuno-reactivity of recombinant AKAP4 protein when probed with anti-AKAP4 antibody pre-neutralized with recombinant AKAP4 protein. Lane 4–7 shows distinct immuno-reactivity against AKAP4 in stage I, II, III, and IV CRC patients positive for anti-AKAP4 antibodies in ELISA. Lane 8–10 shows distinct immuno-reactivity against AKAP4 in WD, MD, and PD histotypes of CRC patients positive for anti-AKAP4 antibodies in ELISA. No immuno-reactivity was seen in the sera of healthy donors (Lane 11–12) or the sera of the CRC patients pre-neutralized with recombinant AKAP4 protein (Lane 13–14). (WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated). (C) Representative images of immunohistochemical analysis shows H&E staining in Stage I, II, III, IV, CRC specimens in top panel. Middle panel shows the immuno-reactivity in the CRC specimens when probed with pre-neutralized anti-AKAP4 antibody. Bottom panel shows no immuno-staining when probed with the sera neutralized with recombinant AKAP4 protein. Original magnification 200×, objective 20×.
AKAP4 gene expression in various stages, grades and histotypes of CRC patients was found in majority (84%) of the CRC patients (Table 1). It is interesting to note that AKAP4 gene expression was significantly associated \( p < 0.026 \) with late stages (III and IV) of CRC patients indicating its potential role in aggressive behavior of the tumor. However, recent report on AKAP4 gene expression in other cancers has revealed only 42% in multiple myeloma, 15 65% in prostate cancer, 16 and 25% in NSCLC. 17 It is important to note that AKAP4 protein expression validation was documented in all the AKAP4 mRNA positive tumor specimens in CRC and other cancers. Yet another testis specific protease (TSP50) was shown to be associated in 68% of CRC patients. It was further documented that expression of TSP50 predicted presence of CRC with 68.4% sensitivity, 92.5% specificity, 95% PPV, and 55.2% NPV. 10 In our study, AKAP4 protein expression, based on IRS, predicted presence of CRC with 84% sensitivity, 100% specificity, 100% of PPV, and 83.33% NPV. Further, TSP50 expression was not shown to be significantly associated with CRC and the clinicopathological features. 10 In contrast, our study revealed that AKAP4 protein expression was significantly associated with late stages (III and IV, \( p < 0.026 \)) of CRC patients suggesting its involvement in spread of cancer cells. Although, majority of early stage (84%, I and II) CRC patients did reveal AKAP4 expression, but no significant association was observed. The present study suggests that AKAP4 may be a potential candidate molecule for diagnosis and therapeutic use for better CRC management which warrants further studies.

Even though, recent studies have well documented that CT antigens are immunogenic and generate humoral response in cancer patients, 4 this is the first report of a humoral response against AKAP4 in majority of CRC patients. In our earlier studies, we reported that AKAP4 elicited immune response in 58% (22 of 38) EOC, 13 79% (72 of 91) breast cancer, 12 and 53% (37 of 70) cervix cancer patients. 14 None of the previous studies have reported humoral response against AKAP4 in CRC patients. Earlier, other investigations have shown humoral response against AKAP4 in small sample size patients, 6 of 19 (32%) in multiple myeloma, 15 10 of 15 (66%) in prostate cancer, 16 and 4 of 17 (23%) in NSCLC. 17 Thus, the present study has put forth an evidence that AKAP4 is highly immunogenic and generates immune response. Our data showed that 82% of the CRC patients expressing AKAP4 demonstrated positive humoral immune response. However, the remaining AKAP4 expressing CRC patients did not have detectable levels of AKAP4 antibodies. It is quite possible that the presence of AKAP4 antibodies may be depended on the genetic makeup of the individual and therefore, physiologically, there may be “patient’s responders” and “patient’s non-responders.” The humoral response against AKAP4 may be suggestive of the presence of the tumor in the patient. Further statistical analysis revealed that antibodies found against AKAP4 in CRC patients predicted presence of malignancy with 81.98% sensitivity, 100% specificity, 100% PPV, and 63.53% NPV. Humoral response against AKAP4 in CRC patients supports potential use as a promising biomarker candidate for early detection and diagnosis and requires further large scale investigation.

Collectively, our data demonstrated that majority of CRC patients exhibit AKAP4 expression and elicit humoral response against AKAP4 protein in the sera irrespective of the stages and the histopathological grades of CRC. AKAP4 expression was associated with late stages of CRC patients. Moreover, majority of CRC patients in this investigation exhibited immune response against AKAP4. Hence, we have put forth an evidence of a possibility of developing serum based biomarker for better cancer management of CRC patients. Further studies are warranted to investigate the association of AKAP4 in large number of CRC patients.

**Material and methods**

**Patient samples**

CRC specimens were obtained during surgical procedure at All India Institute of medical Sciences (AIIMS), New Delhi. The study was carried out according to the guidelines of the ethical committee of the Hospital with their approval. The duly signed consent forms were obtained from the patients prior to the study. Two independent hospital pathologists carefully reviewed and confirmed the diagnosis of CRC specimens. In this study, we investigated two hundred CRC specimens, including 8 patients of stage I, 37 patients of stage II, 104 patients of stage III, and 51 patients of stage IV. In addition, 160 matched available adjacent non-cancerous tissue (ANCT) specimens were also collected. Tumor specimens were collected in 5% buffered formaldehyde prepared in diethylpyrocarbonate (DEPC) water and examined for AKAP4 protein and gene expression employing IHC and *in situ* RNA hybridization studies. In addition, a portion of CRC specimens were also collected in RNAlater (Sigma-Aldrich, St. Louis, MO) and stored at −80°C until use for AKAP4 gene expression analysis. Sera samples from 172 CRC patients available and from 138 healthy donors were also collected to study humoral response. Since it is not study to undertake clinical trials therefore, it was not registered at central registration clinicaltrials.gov.

**RT-PCR**

Total RNA from patient tumor specimens and ANCT was isolated using RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) as per manufacturer’s protocol. The RNA was reverse transcribed using a set of primers and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) as described earlier. 13 Following AKAP4 specific primers were used: AKAP4 Forward primer 5’-TGATACTACAT- GATGTCGATGAT-3’, AKAP4 Reverse primer 5’-GGAC- TAGCAGCATCCCTGTAATCTTTATC-3’, β-actin was used as an internal control to check the quality of cDNA synthesis with following primers: β-actin Forward primer 5’-ATCTGGCACCACACCTTCTCACAATGAGCTGGC-3’, β-actin Reverse primer 5’-CGTCTAGACTCTCCTGGCTGTCCATCAGATCTGC-3’. The PCR products were electrophoresed on 2% agarose gel and photographed under UV light in EC3 Imaging System (UVP, Upland, CA). The amplicons of AKAP4 thus obtained were sub-cloned into TOPO vector (Invitrogen, Carlsbad, CA) to confirm the sequence of the AKAP4. β-Actin mRNA expression was checked as an internal control.
RNA in situ hybridization analysis
For cell type specific expression, serial CRC sections were subjected to in situ RNA hybridization experiments as described earlier. In-vitro synthesis of riboprobes was done using T7 (antisense riboprobe) or T3 (sense riboprobe) RNA polymerases respectively and DIG RNA Labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) respectively and used to hybridize AKAP4 transcript. The hybridized product was visualized using nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indoly-phosphate [(NBT/BCIP) as substrate, Roche Diagnostics GmbH, Mannheim, Germany] and images were captured using Nikon Eclipse E400 microscope (Nikon, Fukuoka, Japan).

Immunohistochemistry
CRC serial sections were H&E stained and were examined to confirm the microscopic foci of CRC by two independent pathologists of the hospitals. Subsequently, AKAP4 immunostaining using serial sections of CRC specimen was performed. The AKAP4 protein expression was detected using anti-AKAP4 antibody or control IgG as described earlier. Briefly, paraffin-embedded tissue sections were blocked with normal goat serum after deparaffinization, rehydration, and endogenous peroxidase removal. Specimens were incubated with anti-AKAP4 antibody or control IgG followed by incubation with goat anti-mouse IgG HRP (Jackson ImmunoResearch Laboratories, West Grove, PA). Serial sections were also checked for the presence of proliferation marker proliferating cell nuclear antigen (PCNA). The immuno-reactivity was visualized using chromogen 0.05% 3, 3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MA).

Immunoreactivity score (IRS)
IRS was calculated as a percentage of cells expressing AKAP4 protein. For determining the IRS, the tissue section slides were reviewed by two independent senior pathologists. More than 500 cells were counted from 5 random fields under 400× magnification. Specimens showing >10% AKAP4 positive cells were considered as positive immuno-reactive specimens.

Humoral response
The circulating anti-AKAP4 antibodies in CRC patients were detected in the sera of the 172 colorectal patients employing ELISA and by Western blotting as described. Neutralization experiments were performed as described earlier to examine the specific humoral response against AKAP4 in CRC patients. Briefly, sera of the CRC patients were pre-incubated with AKAP4 recombinant protein (15μg/mL) and subsequently, were used for probing recombinant AKAP4 by Western blotting as described earlier.12

Statistical analysis
Statistical data analyses were performed using SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA). The statistical difference of AKAP4 gene and protein expression among various tumor stages and histopathological classification was examined using Mann-Whitney U-test. The statistical significance between the humoral response in CRC patients and healthy donors was assessed by unpaired Mann-Whitney U-test. Mann-Whitney U-test was also performed to find the difference between lymph node positive and negative CRC patients. The Pearson’s χ² test was used to assess the association of AKAP4 expression and humoral response among various CRC stages. The Kruskal–Wallis test was used to analyze the difference in AKAP4 expression and humoral response among different CRC stages (I, II, III, IV) and various tumor grades (well differentiated, moderately differentiated, and poorly differentiated). A p value of less than 0.05 was considered statistically significant. The diagnostic accuracy of the tests was evaluated by using Bayesian statistics such as sensitivity [TP/(TP + FN)], specificity [TN/(TN + FP)], PPV [TP/(TP + FP)], and NPV [TN/(TN + FN)] where abbreviations stand for, True Positive (TP), True Negative (TN), False Negative (FN), and False Positive (FP) cases for AKAP4 protein expression and anti-AKAP4 antibodies in control and tumor patients.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

Author’s contributions
NJ, DP, NG, SA, AS, and RF carried out all the experiments, prepared figures and drafted the manuscript. NJ, NKL, AG, and VS participated in data analysis and interpretation of results. AG and VS pathologists performed histopathology examination of all the clinical specimens used in this investigation. RK senior surgeon provided clinical samples and clinico-pathological data from the hospital for this study. AS designed the study, participated in data analysis and interpretation of results. All authors read and approved the manuscript. We acknowledge Dr. V. Kumar, Senior Staff Scientist, International Centre for Genetic Engineering and Biotechnology, New Delhi, India for critical reading and editing of this manuscript.

Funding
This work is supported by grants from Indo-UK Cancer Research Program (Grant No. BT/IN/UK/NII/2006), Centre for Molecular Medicine (Grant No. BT/PR/14549/MED/14/1291), NII-core funding, Department of Biotechnology, Government of India. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References
1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2015. CA Cancer J Clin 2015;65:5-29; PMID:2559415; http://dx.doi.org/10.3322/caac.21254
2. Bian B, Mongrain S, Cagnot S, Langlois M, Boulanger J, Bernatchez G, Carrier JC, Boudreau F, Nathalie R. Cathepsin B promotes colorectal tumorigenesis, cell invasion, and metastasis. Mol Carcinog 2015; PMID:25808857; http://dx.doi.org/10.1002/mc.22312
3. Li M, Yuan Y, Han Y, Liu YX, Yan L, Wang Y, Gu J. Expression profile of cancer-testis genes in 121 human colorectal cancer tissue and adjacent normal tissue. Clin Cancer Res 2005; 11:1809-14; PMID:15756003; http://dx.doi.org/10.1158/1078-0432.CCR-04-1365
4. Suri A, Saini S, Sinha A, Agarwal S, Verma A, Parashar D, Singh S, Gupta N, Jagadish N. Cancer testis antigens: A new paradigm for cancer therapy. Oncoimmunology 2012; 1(7):1194-6; PMID:23170277; http://dx.doi.org/10.4161/onci.20686
5. Otavia LC, Yao TC. Cancer/testis (CT) antigens: Potential targets for immunotherapy. Cancer Sci 2009; 100:2014-21; PMID:19719775; http://dx.doi.org/10.1111/j.1349-7006.2009.01303.x

6. Kanoja D, Garg M, Gupta S, Gupta A, Suri A. Sperm-associated antigen 9 is a novel biomarker for colorectal cancer and is involved in tumor growth and tumorigenicity. Am J Pathol 2011; 178:1009-20; PMID:21356354; http://dx.doi.org/10.1016/j.ajpath.2010.11.047

7. Luo B, Yun X, Fan R, Lin Y, He S, Zhang Q, Mo F, Chen F, Xiao S, Xie X. Cancer testis antigen NY-ESO-1 expression and serum immunogenicity in colorectal cancer: its relationship to clinicopathological parameters. Int J Clin Exp Pathol 2013; 6(12):2835-45; PMID:24294369

8. Jungbluth AA, Chen YT, Stockert E, Busam KJ, Kolb D, Iversen K, Coplan K, Altorki N, Old LJ. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. Int J Cancer 2001; 92:856-60; PMID:11351307; http://dx.doi.org/10.1002/jic.1282

9. Jungbluth AA, Busam KJ, Kolb D, Iversen K, Coplan K, Chen YT, Spagnoli GC, Old LJ. Expression of MAGE-antigens in normal tissues and cancers. Int J Cancer 2000; 85:460-5; PMID:10699915; http://dx.doi.org/10.1002/(SICI)1097-0215(20000215)85:4%3c460::AID-IJC3%3e3.0.CO;2-N

10. Zheng L, Xie G, Duan G, Yan X, Li Q. High expression of testes-specific prostate 50 is associated with poor prognosis in colorectal carcinoma. PLoS One 2011; 6(7):e22203; PMID:21765952; http://dx.doi.org/10.1371/journal.pone.0022203

11. Mohapatra B, Verma S, Shankar S, Suri A. Molecular cloning of human testis mRNA specifically expressed in haploid germ cells, having structural homology with the A-kinase anchoring proteins. Biochem Biophys Res Commun 1998; 244:540-5; PMID:9514854; http://dx.doi.org/10.1006/bbrc.1998.8079

12. Saini S, Jagadish N, Gupta A, Bhatnagar A, Suri A. A novel cancer testis antigen, a-kinase anchor protein 4 (AKAP4) is a potential biomarker for breast cancer. PLoS One 2013; 8(2):e57095; PMID:23451156; http://dx.doi.org/10.1371/journal.pone.0057095

13. Agarwal S, Saini S, Parashar D, Verma A, Sinha A, Jagadish N, Batra A, Suri S, Gupta A, Ansari AS et al. The novel cancer-testis antigen A-kinase anchor protein 4 (AKAP4) is a potential target for immunotherapy of ovarian serous carcinoma. OncoImmunology; 2013; 2: e24270-8; PMID:23762804; http://dx.doi.org/10.4161/onci.24270

14. Agarwal S, Saini S, Parashar D, Verma A, Jagadish N, Batra A, Suri S, Bhatnagar A, Gupta A, Ansari AS et al. Expression and humoral response of a-kinase anchor protein 4 in cervical cancer. Int J Gynecol Cancer 2013; 4:650-8; PMID:23478221; http://dx.doi.org/10.1097/IGC.0b013e31828a6098

15. Chiriva-Internati M, Ferrari R, Yu Y, Hamrick C, Gagliano N, Grizzi F, Frezza E, Jenkins MR, Hardwicke F, D'Cunha N et al. AKAP-4: a novel cancer testis antigen for multiple myeloma. Br J Haematol 2012; 140:464-74; PMID:18217892; http://dx.doi.org/10.1111/j.1365-2141.2007.06940.x

16. Chiriva-Internati M, Yu Y, Mirandola L, D'Cunha N, Hardwicke F, Cannon MJ, Cobos E, Kast WM. Identification of AKAP-4 as a new cancer/testis antigen for detection and immunotherapy of prostate cancer. Prostate 2012; 72:12-23; PMID:21520158; http://dx.doi.org/10.1002/pros.21400

17. Mirandola L, Figueroa JA, Phan TT, Grizzi F, Kim M, Rahman RL, Jenkins MR, Cobos E, Jumper C, Alalawi R et al. Novel antigens in non-small cell lung cancer: SP17, AKAP4, and PTG1 are potential immunotherapeutic targets. Oncotarget 2015; 6(5):2812-26; PMID:25739119; http://dx.doi.org/10.18632/oncotarget.2802

18. Gold MG, Lygren B, Dokurno P, Hoshi N, McNamachie G, Taskén K, Carlson CR, Scott JD, Barford D. Molecular basis of AKAP specificity for PKA regulatory subunits. Mol Cell 2006; 24:383395; PMID:17081989; http://dx.doi.org/10.1016/j.molcel.2006.09.006

19. Scudellari M. A ballsy search for cancer targets. Nat Med 2011; 17:916-8; PMID:21818078; http://dx.doi.org/10.1038/nm0811-916

20. Garg M, Chaurasiya D, Rana J, Jagadish N, Kanoja D, Dudha N, Kamran N, Salhan S, Bhatnagar A, Suri S et al. Sperm-associated antigen 9, a novel cancer testis antigen, is a potential target for immunotherapy in epithelial ovarian cancer. Clin Cancer Res 2007; 13:1421-8; PMID:17332284; http://dx.doi.org/10.1186/1078-0432.CCR-06-2340

21. Garg M, Kanoja D, Khosla A, Dudha N, Suri S, Chaurasiya D, Jagadish N, Seth A, Kumar R, Gupta S et al. Sperm-associated antigen 9 is associated with tumor growth, migration, and invasion in renal cell carcinoma. Cancer Res 2008; 68:8240-8; PMID:18922895; http://dx.doi.org/10.1158/0008-5472.CAN-08-1708

22. Garg M, Kanoja D, Suri S, Gupta A, Suri A, Bhatnagar A, Suri A. Sperm-associated antigen 9: a novel diagnostic marker for thyroid cancer. J Clin Endocrinol Metab 2009; 94:4613-8; PMID:19820019; http://dx.doi.org/10.1210/jc.2009-0703

23. Garg M, Kanoja D, Salhan S, Suri S, Gupta A, Lohiya NK, Suri A. Sperm-associated antigen 9 is a biomarker for early cervical carcinoma. Cancer 2009; 115:2671-83; PMID:19326449; http://dx.doi.org/10.1002/cncr.24293

24. Garg M, Saini S, Parashar D, Verma A, Sinha A, Jagadish N, Batra A, Suri S, Gupta A, Ansari AS et al. The novel cancer-testis antigen a-kinase anchor protein 4 in cervical cancer. Int J Gynecol Cancer 2013; 23:383-9; PMID:23490577; http://dx.doi.org/10.1002/ijg.22432

25. Kanoja D, Garg M, Saini S, Agarwal S, Parashar D, Jagadish N, Seth A, Bhatnagar A, Gupta A, Kumar R et al. Sperm-associated antigen 9 plays an important role in bladder transitional cell carcinoma. PLoS One 2013; 8(12):e81348; PMID:24349057; http://dx.doi.org/10.1371/journal.pone.0081348