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# Genetic Characterization and Phylogenetic Analysis of CEA-CAM1 Gene Immunodominant Region from a Case of Canine Mammary Tumour

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## A B S T R A C T

Canine Mammary Tumour (CMT) is the most common malignancy of unspayed female dogs and is of potential importance as a model for human breast cancer. Mortality rate in female dogs is three times than that of human females with breast cancer. Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) also called as CD66a (Cluster of Differentiation 66a), is a glycoprotein which is over expressed in various human cancers. Although several studies have shown correlation of gene expression with tumour progression, metastasis and overall survival in human breast cancer, very few studies have focussed so far on CEAM 1 gene and its expression in canine mammary cancers. Therefore, present study was undertaken to determine CEA-CAM1 gene sequence in a clinical case of canine mammary tumour (CMT), showing over expression of the gene and showing presence of anti-CEA autoantibodies in the serum. C-terminal immunodominant region of CEA-CAM1 gene(from case of canine mammary tumour) was amplified and cloned in prokaryotic expression vector. Further the gene was also sequenced. Alignment of CEA-CAM1 mRNA sequence from CMT tissue and normal healthy dog tissue showed 100% similarity between them showing absence of any mutation in CEA-CAM1 coding region in case of CMT suggesting that mechanisms other than gene alterations and mutations are responsible for over expression. Some possible mechanisms for production of autoantibodies against CEA-CAM1 could be due to increased antigenic load due to its over expression in tumour tissues and reduced degradation of protein etc. Phylogenetic analysis of the dog CEA-CAM1 gene showed that dog gene sequence has more than 88% nucleotide sequence identity with *Mustela putorius* (European polecat), *Ursus maritimus* (polar bear), *Callorhinus ursinus* (Northern fur seal) and Pantherapardus (leopard), whereas canine CEA-CAM1 sequence is 74.5% identical with Homo sapiens gene.

## Introduction

The persistence of mammary cancer is different in various domesticated animals. Dog is the most commonly affected domesticated species with cancer, having a prevalence more than three times than that in human females (Schneider, 1970). Canine mammary tumour (CMT) is the most common cancer of female dogs exceeding 5 years of age (Davidson, 2003; Murphy, 2008; Salas *et al.,* 2015), accounting above 40% of all tumour diagnosed (Sleeckx *et al.,* 2011; Beck *et al.,* 2013). About half of the mammary cancers are benign or localised that can be treated by surgery alone but half are malignant tumours which have the potency to spread in other parts of body, resulting in high death rates of bitches. In dogs, mammary tumours occurs mostly in unspayed female dogs accounting for about 52% of all neoplasms (Macewen Withrow, 1996). The mortality rates in dogs suffering from canine mammary tumour (CMT) are more than three times than that of human mammary cancer (Egenvall *et al.,* 2005; Shafiee *et al.,* 2013). Thus CMT represents an important malignancy of dogs which provide ample scope for formulation of new methods for disease control and management.

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEA-CAM1) is a glycosylated cell surface protein which belongs to the immunoglobulin (Ig) gene superfamily (Zhou *et al.,* 2001; Hoskovec *et al.,* 2012). It helps in cell adhesion, apoptosis, intracellulalar signalling and tumour progression. Its overexpression suggest immune dysfunction and progression of many epithelial cancers. CEA is one of the first biomarker to be identified in human breast cancer (Lian, *et al.,* 2019). It is routinely measured in serum but can also be found in tissues using various immunochemical methods like electrochemistry, Immunohistochemistry (IHC), radio-immunological methods (RIA) and luminescence immunoassay (ECL*)* (Ledecky *et al.,*2013*;*Shao *et al.,*2015*).*

Certain tumours overexpresses certain proteins called as tumour associated antigens in due course of time. These tumour associated antigens are considered as foreign by the host immune system as the immune system expresses anti-tumour immune response against them. Although these antigens are normal proteins involved in cell cycle and cellular pathways but there is production of auto–antibodies against these tumour associated antigens. Since CEA-CAM1 is highly overexpressed in CMT cases and anti-CEA-CAM1 autoantibodies have also been found in CMT cases in high frequency therefore the present study was undertaken to determine CEA-CAM1 gene sequence in a clinical case of canine mammary tumour (CMT) (which showed anti-CEA-CAM1 autoantibodies in serum) and compare it with the sequence of CEA-CAM1 of healthy, non-cancerous dog.

## Materials and Methods

### Collection of tumour tissues and serum samples

Canine mammary tumour tissues and serum samples used in the study were collected following surgical excision of tumour from clinical cases of CMT referred for surgery to the ``Referral Veterinary Polyclinic", ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, India. The tissues were fixed in 10% Neutral buffered formalin (NBF). Thereafter the tissues were paraffin embedded and 5µm sections were cut for histopathological examination. The sections were stained by haematoxylin and eosin (H&E) stain and examined under microscope (Goldschimdt *et al.,* 2011).

## Isolation of RNA and synthesis of cDNA

RNA was isolated from CMT tissue sample by Trizol method as per the manufacturer's protocol. Quantification of RNA was done by using Qubit RNA BR Assay Kit (Invitrogen, USA). Preparation of cDNA from total RNA was carried out by Revert Aid cDNA synthesis kit (Fermentas, USA) using Oligo (dT) primers, dNTPS, RNAase inhibitor, total RNA and reverse transcriptase in a totalvolume of 20 µl. The prepared cDNA was stored at -80° for further use.

## Amplification of the CEA-CAM1 gene

Primers were designed for amplification of 851 bp region for CEA-1gene by using Premier 5.0 software (National Bioscience), analysed using oligoanalyser 3.1 and custom synthesised by Integrated DNA technologies (USA). The PCR was performed by Kapa Hi-fidelity PCR master mix (2X) with a total of 50 µl reaction mixture as per the manufacturer's protocol. The reaction mixture contained25 µl master mix, 1 µl of 20 pM each of forward and reverse primer and 3 µl of template cDNA. Annealing temperature was optimised using gradient PCR machine (VeritiThermocycler, Applied biosystems). The, annealing temperature optimized by gradient PCR followed by temperature range between 53° - 61°.

## Cloning of the CEA-CAM1 gene in prokaryotic expression vector

The amplified PCR product was gel purified by using min-elute PCR purification kit (Qiagen, Hilden, Germany) as per manufacturer`s protocol. PCR product and pH6HTN His 6 HaloTag T7 Vector (Promega, Madison) were digested withXBA1 and APA1restriction endonucleases (NEB, England) at 37° for 2 hrs to create complementary overhangs. The forward and reverse primers used for amplifying the CEA-CAM1gene were having the site for XBA1 and APA1 restriction endonucleases. The XBA1 and APA1 digested purified PCR product were ligated with RE digested pH6HTN vector by using T4DNA ligase (Promega, Madison) and were transformed into *E.coli* KRX competent cells. Transformed cells were spread on LB Agar plates containing 100 µl /ml ampicillin for bacterial colonies appearance containing recombinant clones. The recombinant clones were identified by colony PCR and were further confirmed by RE analysis and sequencing. The recombinant plasmids were subjected to RE digestion using XBA1 and APA1 restriction endonucleases.

## Phylogenetic Analysis

Identification of sequences similar to the sequences of the isolated CEA-CAM1 gene were performed by BLAST against NCBI nucleotide database. The sequences were aligned by Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm in MEGA7 software (REF). The phylogeny was then inferred by maximum-likelihood method upon assessment of best fit substitution model.

## Histopathology and immunohistochemistry

Histopathological classification of paraffin embedded tumour tissue sections was performed as per Goldschmidt *et al.,* (2011). Overexpression of CEA-CAM1 in the parrafin embedded CMT sections was confirmed by immunohistochemistry (IHC). Sections were covered with 100-150µl of 1:50 diluted rabbit anti-cea antibody. The negative controls were covered with PBS only. The slides were incubated in humidified chamber overnight at 4℃. The slides were washed thrice with PBS and then incubated with 1:200 diluted anti rabbit secondary antibody (Sigma, USA) at 37℃ for 1 hour in humidified chamber. The

slides were washed thrice with PBS and DAB (Sigma, USA) was added to the tissue sections for 2 min. Once the colour starts developing the reaction was stopped immediately by washing slides in distilled water. The sections were then counter stained by Mayer`s haematoxylin (Sigma, USA) for 3-5 minutes and finally rinsed in running tap water for 5 min, air dried, cleared in xylene and mounted in DPX.

## Results and Discussion

CEA is well established as a therapeutic and diagnostic biomarker in human breast cancer. The aim of comparative oncology is to speed up developments for both, human and companion animal cancer patients. Since canine mammary tumour shares many similarities with human breast cancer, carcinoembryonic antigen could be a therapeutic target not only for human but also for canines. In human cancer patients, overexpression of CEACAM 1 have been reported. Studies conducted in our laboratory also evidenced presence of anti-CEACAM1 autoantibodies in sera samples of dogs with canine mammary cancer. Therefore in the present study tumour tissue was collected from a case of canine mammary tumour showing presence of CEACAM1 autoantibodies in the serum.

The histopathological analysis of CMT revealed that the tissue was complex carcinoma as per Goldschimdt *et al.,* 2011. Figure 1A and 1B shows gross appearance and microscopic image of H&E stained CMT tissue. The tissue was used for RNA isolation and amplification of CEA-CAM1 gene. The tumour tissue showed over expression of CEA-CAM1 upon Immunohistochemical staining using CEA-CAM1 specific antibodies as shown in Figure 1C.As explained earlier, the 851bp c-terminal immunodominant region of CEA-CAM1 gene was amplified by PCR and the amplified gene showed expected band size of 851 bp upon agarose gel electrophoresis (Fig 2). Cloning of amplified region of CEA-CAM1gene was done in pH6HTNHis6 Halo Tag T7 prokaryotic expression vector (Promega) and transformed cells were spread on LB Agar plates for appearance of bacterial colonies containing recombinant clones. The recombinant colonies showed amplification of CEACAM1 gene by PCR as shown in figure 3A. The recombinant clones were further confirmed by RE analysis and sequencing. RE digestion of plasmid isolated from recombinant colony was performed using XBA1 and APA1 restriction endonucleases and insert of size corresponding to CEA-CAM1 gene fragment (851 bp) was released from the vector as shown in Figure 3B.The recombinant plasmid was confirmed by plasmid DNA sequencing.

The alignment of the CEA-CAM1 gene sequence (from CMT case) showed 100% similarity with healthy dog`s (*Canislupus familaris*) CEA-CAM1 gene sequence (NM_001097557) available in the GenBank database. These findings showed absence of any mutation in the CEA-CAM1 gene coding region in canine mammary tumour case.

BLAST analysis of the c-terminal immunodominant region, CEA-CAM1 showed greater than 70% similarity with sequence from other common animal species in the Gen Bank database showing conserved nature of the protein. The results from BLAST analysis are compiled in Table 1. The dog`s CEA-CAM1 mRNA sequence showed more than 88% nucleotide sequence similarity with *Mustela putorius* (European polecat), *Ursus maritimus* (polar bear), *Callorhinus ursinus* (Northern fur seal) and *Panthera pardus* (leopard). The canine CEA-CAM1 sequence has 74.5%% similarity with Homo sapiens gene.

**Table.1** Percent identity of CEACAM1 gene sequence from a case of canine mammary tumour with other animal species

| Scientific name | Common Name | Accession No | Percent identity (%) |
|---|---|---|---|
| ***Canis lupus familiaris*** | Dog | NM_001097557.1 | 100 |
| ***Callorhinus ursinus*** | Northern fur seal | XM_025849450.1 | 89.98 |
| ***Ursus maritimus*** | Polar bear | XM_040634034.1 | 89.24 |
| ***Mustela putorius*** | European polecat | XM_004780425.2 | 88.45 |
| ***Panthera pardus*** | leopard | XM_019434422.1 | 88.19 |
| ***Equus caballus*** | Horses | MF564057.1 | 86.08 |
| ***Gorilla gorillagorilla*** | gorilla | XM_031004080.1 | 78.82 |
| ***Rhinopithe cusroxellana*** | Hubei Golden Snub-nosed Monkey | XM_010358723.2 | 78.44 |
| ***Chinchilla lanigera*** | Chinchilla | XM_005412355.1 | 78.46 |
| ***Macacanemestrina*** | Sunda Pig-tailedMacaque | XM_011764531.2 | 78.09 |
| ***Macaca mulatta*** | rhesus macaque | XM_015124068.2 | 77.15 |
| ***Callithri xjacchus*** | White-tufted-ear Marmoset | XM_002762196.2 | 77.13 |
| ***Mus musculus*** | house mouse | NM_001039185.1 | 77.31 |
| ***Rattus norvegicus*** | brown rat | BC061740.1 | 76.56 |
| ***Homo sapiens*** | human | BC024164.1 | 74.45 |
| ***Sus scrofa*** | pigs | XM_005655889.2 | 80.98 |
| ***Marmota marmotamarmota*** | Alpine Marmot | XM_015496802.1 | 70.36 |
| ***Bubalus bubalis*** | water buffalo | XM_006041226.2 | 75.63 |
| ***Bostaurus*** | Cattle | NM_205788.1 | 74.96 |
| ***Bison bisonbison*** | American bison | XM_010842547.1 | 74.47 |

**Fig.1** Phylogenetic analysis showing similarity of the dog CEA-CAM1 gene with various animal species.

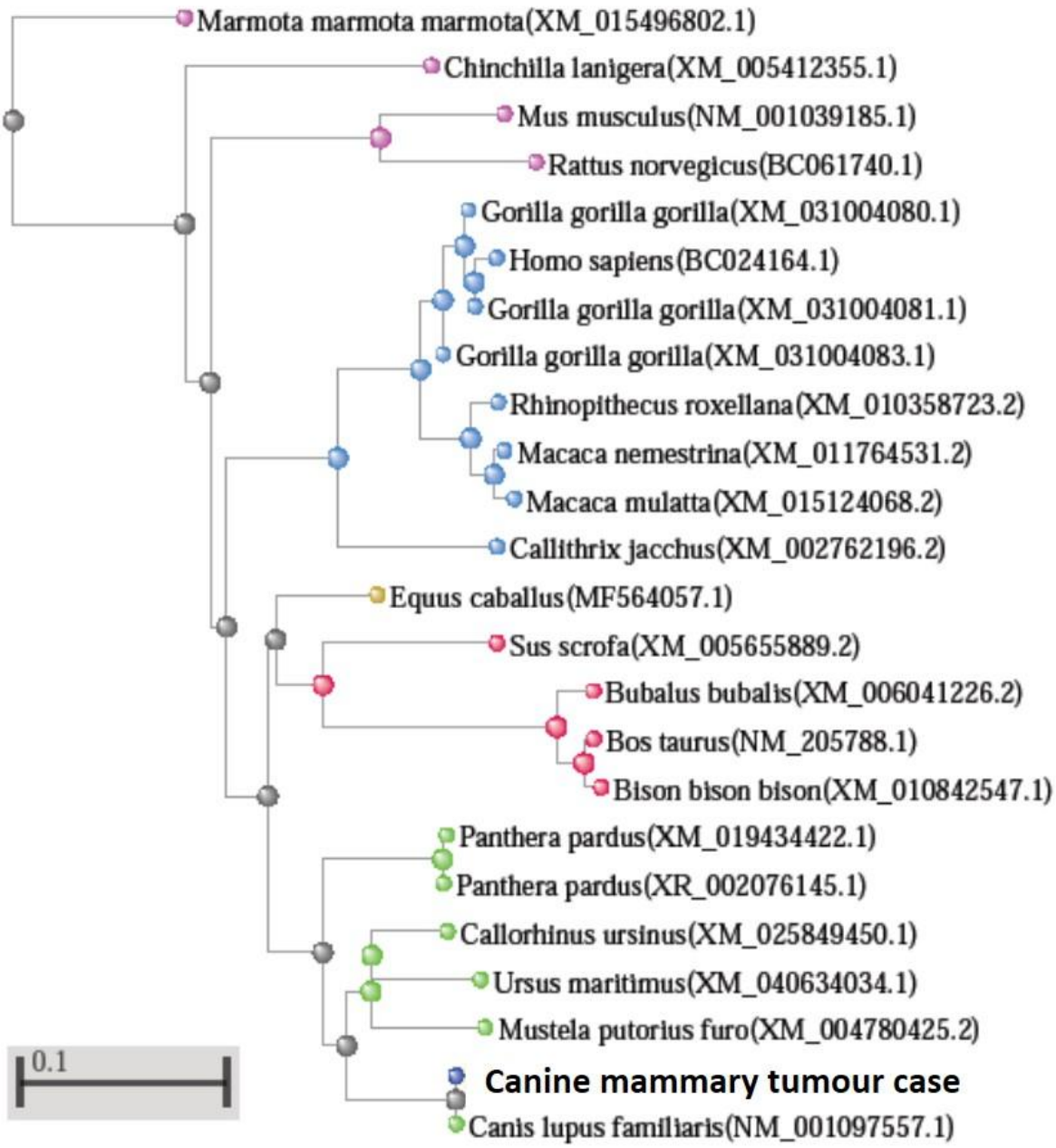


**Fig.2** Gross (A), Histopathological (B) and Immunohistochemical(C) appearance of tumour tissues used for CEA-CAM1gene amplification. Histopathological analysis of H&E stained tumour tissue revealed that the tumour was complex type carcinoma. Immunohistochemistry revealed overexpression of CEACAM1 in CMT tissue.

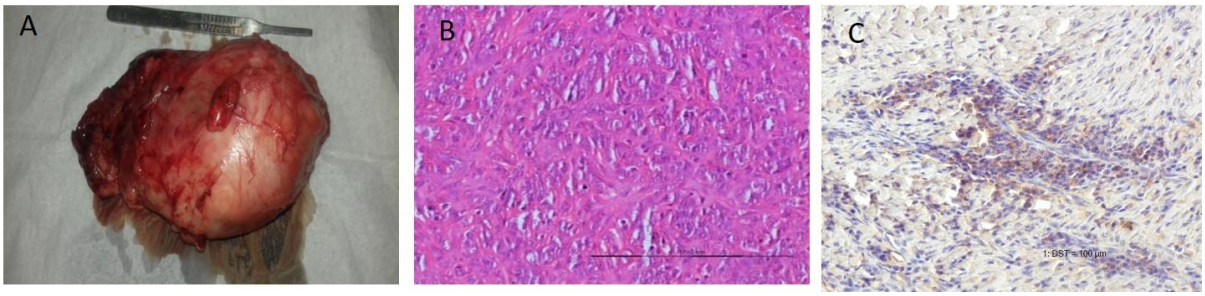



**Fig.3** Amplification of CEA-CAM1 gene from CMT tissue.

a. Lane (M) 1KB DNA ladder. Lane (1) Amplified 851 bp CEA-CAM1 gene PCR product

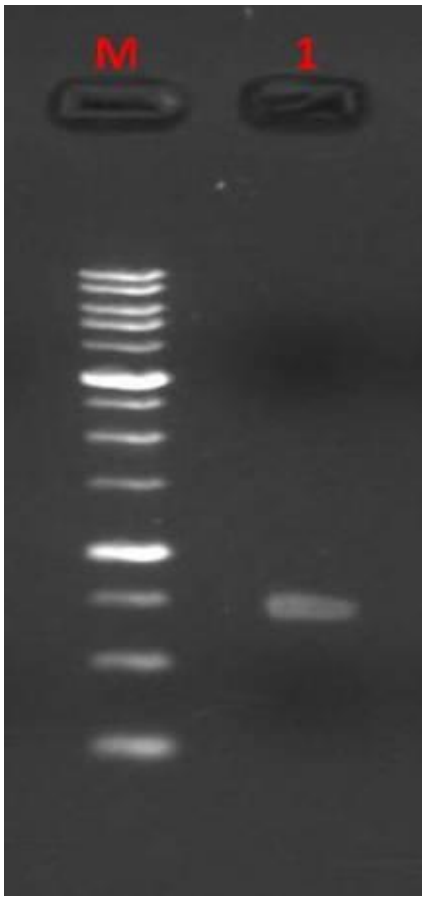
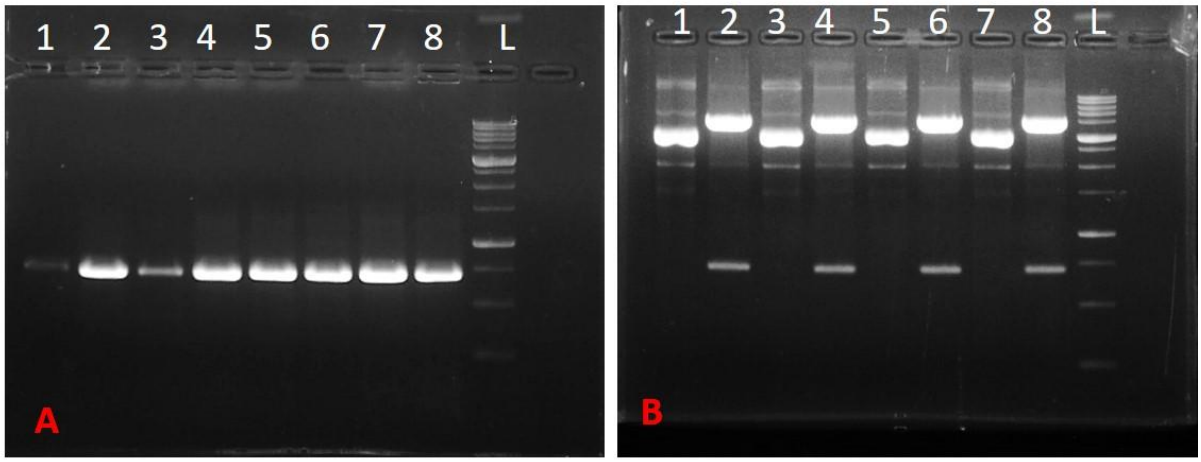


**Fig.4** Cloning of CEA-CAM1 gene in prokaryotic expression vector -
(A) Confirmation of recombinant clones by colony PCR
Lanes 1-8 showing amplification of CEA-CAM1 gene from recombinant colonies. Lane L, 1kb DNA ladder.
(B) Confirmation of recombinant clones by RE digestion
Lanes 2, 4,6, 8 showing release of fragment corresponding to size of CEA-CAM1 gene from the recombinant plasmids, upon RE digestion. Lanes 1, 3, 5, 7 showing no release of fragment corresponding to size of CEA-CAM1 gene from the non-recombinant plasmids. Lane L, 1kb DNA ladder




The aim of the present study was to determine whether any mutations exist in the sequence of CEACAM1gene in the dogs with canine mammary tumour and showing overexpression of gene with high-titre CEACAM1 autoantibodies. For this canine mammary tumour tissues were collected from dogs showing serum autoantibodies to CEACAM1 as determined by Indirect ELISA. The 851 bp c-terminal immunodominant region of CEA-CAM1gene was amplified from canine mammary tumour case (which also showed overexpression of CEACAM1 gene by IHC) and cloned in prokaryotic expression vector. Analysis of 851bp CEA-CAM1 gene fragment in case of canine mammary tumour revealed absence of any mutation in this region as compared to CEA-CAM1 gene sequence of a non-cancerous healthy dog available in Genbank database. Phylogenetic analysis revealed close similarity of the gene with other animal species available in the Gen bank database.

CEA-CAM1 is expressed in normal quantity in normal cells but is overexpressed in cancerous cells. Besides overexpression anti-CEA-CAM1 autoantibodies have been found to be present in sera of dogs suffering from CMT as compared to normal, healthy dogs. Therefore, the present study focussed whether these autoantibodies are produced due to mutated CEA-CAM1 gene or mutated CEA-CAM1 protein in clinical case of CMT. However in the study, no mutation is being found in CEA-CAM1 gene coding region in case of CMT. This indicates that mechanisms other than gene alteration or gene mutation are responsible for production of these autoantibodies. These autoantibodies are not produced by mutated gene or protein but possibly due to increased antigenic load owing to overexpression of CEA-CAM1 and its reduced degradation. Several other theories

such as alteration in antigen expression, altered expression or presentation of antigen, loss of tolerance, post translational modification and abnormal location etc have been put forward for production of autoantibodies in cancerous cells (Zaenker *et al.,* 2016).

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## Conflict of interest

Authors of this manuscript declare no conflict of interest.

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