ORIGINAL ARTICLE

The clinical and biological implications of N-WASP expression in human colorectal cancer

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ABSTRACT

Backgrounds: Neural Wiskott-Aldrich Syndrome protein, N-WASP, a member of the WASP family proteins is a regulator of ARP2/3 and cytoskeleton in the cells and has been implicated in regulating cell motility and morphology. N-WASP has been implicated in the development and progression of certain solid tumours. In the present study, we initially investigated the expression levels of N-WASP in a cohort of human colorectal cancers and explored the relationship between N-WASP and clinical outcome. We further examined the impact of N-WASP on the biological functions of colon cancer cells.

Materials and methods: A cohort of fresh frozen human colon tissues were used. N-WASP protein in tissues was analysed using an immunohistochemical method. N-WASP transcripts in the tissues were quantified using real-time quantitative PCR methods and correlated with clinical and pathological information of the patients together with clinical outcome. Human colon cancer cell line, HRT18, weakly positive for N-WASP was genetically modified to either over-express N-WASP or to lose N-WASP expression by way of ribozyme transgenes. Cell functions were determined after the genetic manipulation.

Results: Colonic epithelial cells stained positive for N-WASP with the staining mainly in the cytoplasmic region of the cells. However, colon tumour cells had greatly reduced N-WASP staining. The reduction of N-WASP protein was well reflected at message level, in that colon tumour tissues had significantly lower levels of N-WASP transcript compared with normal tissues (P<0.001). Significantly lower levels of N-WASP transcript were seen in node positive tumours and tumour with muscular invasion. Patients with low levels of N-WASP transcript had short overall and disease free survivals. Over-expression of N-WASP markedly reduced the adhesion, cellular motility and invasiveness of HRT18 cells. Likewise, knocking down N-WASP resulted in an increase in matrix adhesion and invasion, which was blocked by FAK (focal adhesion kinase) inhibitor.

Conclusions: N-WASP expression is aberrant in human colon cancer. A reduction of N-WASP in colon tumours is associated with disease progression and a poor clinical outcome of the patients. In addition, N-WASP expression in colon cancer cells is inversely correlated with the aggressiveness of the cells, namely adhesion and invasiveness. Collectively, this study indicates that N-WASP carries the hallmark of a tumour metastasis suppressor in human colon cancer. This is likely to be via a FAK mediated pathway.

KEY WORDS N-WASP; colon cancer; survival; prognosis; metastasis; cell migration; FAK

Introduction

Colorectal cancer is one of the most commonly seen cancer throughout the world and is one of the most prevalent tumours in Western countries, including the United Kingdom. In the UK, colorectal cancer is the second and third most common cancers counting for 12% and 13% in females and males, respectively (1,2). Perhaps the most life threatening event in colorectal cancer is the regional and systemic spread of cancer cells, subsequently forming metastatic lesions. Apart from the

traditionally routes of metastasis, namely lymphatic and systemic spread, colon cancer cells frequently have peritoneal spreading after penetrating the muscular layers and serious membrane. Together, locally and systemically advanced diseases are seen in approximately 20% of the patients. Spreading of cancer cells in the body, metastasis, is controlled by a number highly relevant but separate steps collectively known as metastatic cascade, which itself is influenced by a number of intrinsic and extrinsic factors in the body and in cancer cells. Factors that are primarily affecting the metastatic process of cancer cells, without influence other functions such as cell growth are generally referred to as metastatic regulating factors including metastatic suppressors (3,4). Classical metastatic suppressors including nm23, BRMS1 and recently, N-WASP, Neural Wiskott-Aldrich Syndrome protein has been suggested to be a potential metastatic suppressor (5-7).

N-WASP is also known as WASL (Wiskott-Aldrich Syndrome Gene-Like) protein belongs to the WAS family (8,9). Other members of the WASP family include Wiskott-Aldrich Syndrome protein family, member-1, also known as WASF-1 or WAVE-1 (WASP family, Verprolin Homology Domain-Containing protein-1), WASP-2 (or WAVE-2), and WASP-3 (or WAVE-3). Wiskott-Aldrich syndrome (WAS) or also described as Werlhof's disease was originally described in American kindred where it was manifested as eczema, thrombocytopenia, proneness to infection, and bloody diarrhea (10). Death of patients with WAS were mainly due to infections or bleeding, but also development of malignancies: lymphoreticular tumors, leukemia reticuloendothelial system malignancies (11). The N-WASP protein is a regulator of actin polymerization by stimulating the actin-nucleating activity of the actin-related protein 2/3 (Arp2/3) complex (12). It has also been shown that the WAS protein functions as a signal transduction adaptor downstream of Cdc42 (13). N-WASP has several functional motifs, such as a pleckstrin homology (PH) domain and cofilin-homologous region, through which N-WASP depolymerizes actin filaments.N-WASP-stimulated actin assembly is responsible for membrane ruffling (14). N-WASP activity is regulated by an intramolecular interaction that is alleviated following concomitant binding of Cdc42-GTP to the Cdc42/Rac interactive binding (CRIB) domain and PtdIns (4,5)P2 to the polybasic region (15). The N-WASP gene encodes a protein which has 505-amino acids. WASP is a key regulator of actin polymerization in hematopoietic cells with 5 domains involved in signalling, cell motility/migration, in immune synapse formation and in facilitating the nuclear translocation of nuclear factor kappaB (13). Mutations of WASP are located throughout the gene and either inhibit or

dysregulate normal WASP function: classic WAS occurs when WASP is absent, X-linked thrombocytopenia when mutated WASP is expressed, and X-linked neutropenia when missense mutations occur in the Cdc42-binding site (13).

Despite the fact that N-WASP has been widely studied in cells including some cancer cells, investigations into the clinical aspect of N-WASP in human cancer are somewhat hard to come by. In human breast cancer, we have shown that N-WASP expression was significantly reduced when compared with normal tissues and this reduction was associated with poor clinical outcome and disease progression of the patients (16). In human oesophageal cancer, there appears to be no significant difference between tumour tissues and adjacent normal tissues (17). There have been no reports on studies of N-WASP into human colon cancer. Here, we report the expression pattern of N-WASP in human cancer and the biological impact of N-WASP on human colon cancer cells.

Materials ahd methods

Tissues, cells and materials

Fresh tissues were collected immediately after surgery. Normal tissues from the same patients were collected from the end of resected bowels and free from cancer cells. The tissues were stored at −80 ℃ until use. Patients were followed up in clinics. Pathological reports and clinical outcomes were recorded and are shown in table 1. All procedures were approved by local ethics committee. Human colon cancer cell line, HRT18 was obtained from ECACC (European Collection of Animal Cell Cultures, Salisbury, England, UK). FAK inhibitor, PF573228 was from Tocris (Bristol, UK). All other materials were from Sigma unless stated otherwise.

Tissue processing and extraction of RNA and generation of cDNA

Fresh frozen tissues were sections using a cryostat (Leica) at 8 μM thickness. A portion of the sections were immediately fixed and used for histology and immunohistological analysis. The remaining portions were combined and used for extraction of total RNA, after homogenised with a hand held homogenizer.

Detection of N-WASP using RT-PCR

Routine RT-PCR was carried out using a PCR master mix that was commercially available (AbGene). Primers were designed using the Beacon Designer software (version 2, California, USA), to amplify regions of human N-WASP that have no

Table 1. Demographic details of the clinical cohort.

Group		No of samples
Tissues	Normal	68
	Tumour	68
Differentiation	Good	2
	Moderate	52
	Poor	14
Nodal status	Negative	37
	Positive	31
TNM staging	TNM1	8
	TNM2	28
	TNM3	26
	TNM4	6
T-staging	T1	2
	T2	9
	T3	39
	T4	18
Dukes staging	Dukes-A	6
	Dukes-B	32
	Dukes-C	30
Clinical outcome	Disease free	27
	With distant metastasis	19
	Died of colon cancer	22

significant overlap with other known sequences and that the amplified products span over at least one intron. The primers used to amplify N-WASP were given in table 2. Reactions were carried out at the following conditions: 94 ℃ for 5 minutes, 36 cycles of 94 ℃ for 15 seconds, 55 ℃ for 25 seconds and 72 ℃ for 15 seconds. PCR products were separated on a 2% agarose gel and photographed using a digital camera mounted over a UV transluminator.

Quantitative analysis of N-WASP

The levels of N-WASP transcripts in cDNA samples from tissues and cells was determined using a real-time quantitative PCR, based on the AmplifluorTM technology, modified from previous reported (18,19). PCR primers are given in table 2 The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which has the Z sequence, 10 pmol of FAM-tagged probe (Intergen Inc), and cDNA from approximate 50 ng RNA. GAPDH and CK19 were used as a house keeping gene and similarly analysed. The reaction was carried out using

IcyclerIQtm (Bio-Rad) which equiped with an optic unit that allows real time detection of 96 reactions, using the following condition: 94 ℃ for 12 minutes, 50 cycles of 94 ℃ for 15 seconds, 55 ℃ for 40 seconds and 72 ℃ for 20 seconds. The levels of the transcripts were generated from a standard that was simultaneously amplified with the samples and normalised to CK19 and GAPDH. They are shown here as the N-WASP/CK19 ratio.

Immunohistochemical staining of N-WASP protein

This procedure was similar to a method previously reported (16). Frozen sections of normal and tumour tissues of colon were mounted on super frost plus microscope slides and fixed in a mixture of 50% Acetone and 50% methanol. The sections were then placed in "Optimax" wash buffer for 5-10 minutes to rehydrate. Sections were incubated for 20 mins in a horse serum containing blocking solution and probed with the primary antibody (anti-human N-WASP, Santa Cruz Biotechnologies Inc., Santa Cruz, California, USA). Following extensive washings, sections were incubated for 30 mins in the secondary

Table 2. Primers used in the present study.

Primer name	Sense primer(5' - 3')	Anti-sense primer(5' - 3')
N-WASP PCR	agtccctcttcactttcctc	agtccctcttcactttcctc
	attttcatacctttgctgga	taacagcttcaacacctcct
N-WASP expression	atgagctccgtccagcag	tcagtcttcccactcatcatc
Anti-NWASP transgenes	ctgcaggagttctttgaccacatacagttccctgatgagtccgtgagga	actagttggtgcagttatatgcagcagatttcgtcctcacggact
	ctgcagacaagcaacaccactgcacttctttctgatgagtccgtgagga	actagttatatgcagcagatcggaactgtatgtggtttcgtcctcacggact
	ctgcaggtgcagctgtgggagctcttctgatgagtccgtgagga	actagtgctaggggaagaggcgctcctcccccaccaccttttcgtcctcacggact
N-WASP Q-PCR	gagctggatgagaacaacac	actgaacctgaccgtacaaaagaagtggcaggaagagt
GAPDH	ggctgcttttaactctggta	gactgtggtcatgagtcctt
GAPDH Q-PCR	ctgagtacgtcgtggagtc	actgaacctgaccgtacacagagatgatgacccttttg
Primers for plasmid	taatacgactcactataggg (t7f)	tagaaggcacagtcgagg (bghr)
For screening transgenes	ctgatgagtccgtgaggacgaa (rbtpf)	ttcgtcctcacggactcatcag (rbbmr)
Actin	atgatatcgccgcgctcg	cgctcggtgaggatcttca

biotinylated antibody (Multilink Swine anti- goat/mouse/rabbit immunoglobulin, Dako Inc.). Following washings, Avidin Biotin Complex (Vector Laboratories) was then applied to the sections followed by extensive washings. Diamino benzidine chromogen (Vector Labs) was then added to the sections which were incubated in the dark for 5 mins. Sections were then counter stained in Gill's Haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a cover slip.

Expression constructs for human N-WASP and anti-N-WASP transgenes

Full length human N-WASP was amplified from normal human mammary cDNA library as we previously reported using expression specific primers shown in table 2 (Martin *et al* 2004). The product was T-A cloned into a pEF6/V5/His-TOPO-TA (Invitrogen) vector. Anti-N-WASP ribozymes were designed based on the predicted secondary structure of human N-WASP (Figure 1A) using short oligos given in table 2. Ribozymes were similarly cloned into the pEF6/V5/His-TOPO-TA vectors. Plasmids with corrected inserted full length N-WASP was electroporated into human colon cancer cell, HRT18 cells. Expression of the gene was confirmed by RT-PCR.

In vitro assays analyzing N-WASP gene transformed breast cancer cells

In vitro invasiness. Invasiveness of HRT18 cells were assessed using the following *in vitro* assay as previously reported

(20). Transwell chambers equipped with 6.5 mm diameter polycarbonate filter (pore size 8 µm) (Becton Dickinson Labware, Oxford, UK) were pre-coated with 50 µg/membrane of solubilised basement membrane in the form of Matrigel (Collaborative Research Products, Bedford, MA). After membrane re-hydration, 15,000 cells were aliquoted into each insert with/without HGF/SF (25 ng/mL). After 96 h co-culture non-invasive cells were removed with cotton swabs. Invaded cells on the underside of the insert were fixed and stained with crystal violet, followed by microscopic counting (20 fields/insert).

Cell growth assay (20). Cells were plated into 96-wells at 3,000 per well. This allowed for 72 hours, after which cells were fixed with 4% formalin before stained with crystal violet (0.5%, w/w). The rate of cells growth was calculated using the absorbance of colour staining.

Cytocarrier based cell motility assay (21,22). A cell motility assay was carried out. Briefly, cells were pre-coated onto cytodex-2 carrier beads (Sigma-Aldrich, Poole, UK) for 2 hours in complete medium. After the medium was aspirated and the cells washed (X2 in complete medium), they were aliquoted into wells of a 96-well plate in triplicate (300 µL/well). HGF (25 ng/mL) was added and the cells incubated over-night. The beads were washed off in medium, and the cells that had migrated onto the floor of the well fixed (4% formaldehyde) and stained with crystal violet. The cells were counted microscopically (X40).Cell-matrix adhesion assay. The cell-matrix attachment assay was carried out as previously reported23. Briefly, Matrigel (1 mg/well) was added to a 96-well plates, which were incubated for
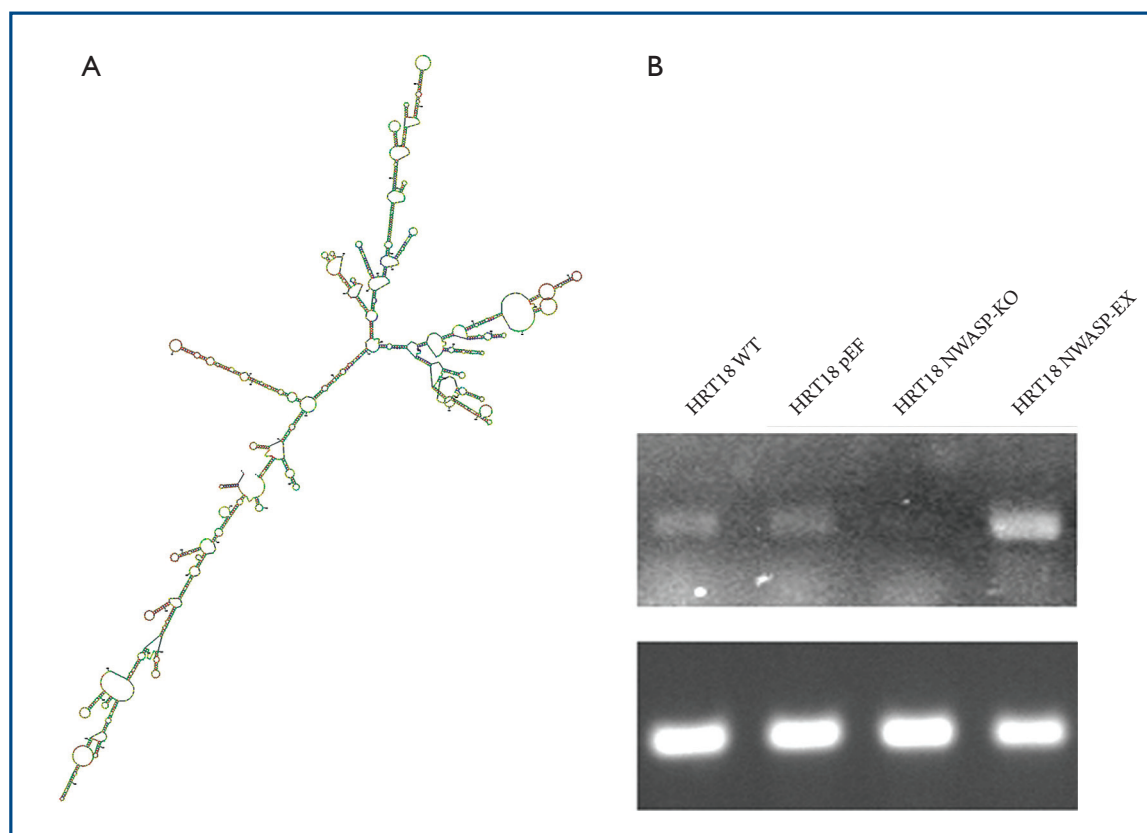
Figure 1. Genetically manipulation of N-WASP expression in human colon cancer cell line. A: The predicted secondary structure of human N-WASP; B: Manipulation of N-WASP expression in HRT-18 cells. HRT18 was weakly positive for N-WASP. Anti-N-WASP transgene successfully knocked down N-WASP expression in the cell and N-WASP expression construct markedly increased level of expression of N-WASP as revealed by RT-PCT.

24 hours to allow binding of matrix protein to the surface of the well. The plates were then washed and blocked with 5% BSA (bovine serum albumin). Cells were added at 10^4/well for 30 minutes, followed by aspiration and washing. The number of attached cells was determined by direct counting under microscope.

Statistical analysis

Statistical analysis was performed by MINITAB version 13.32 (Minitab Inc. State College, PA, USA) using a two-sample student t-test and the non-parametric Mann-Whitney confidence interval and test, where appropriate. Statistical analysis was carried out using Mann-Whitney U test and the Kruskal-Wallis test for tissue samples. Patients long term survival was analysed using Kaplan-Meier methods with SPSS (version SPAW18) package.

Colon epithelial cells expressed N-WASP, which was reduced in cancer cells

Normal colon epithelial cells stained strongly for the N-WASP protein. This is primarily seen in the cytoplasmic region of the cells (Figure 2A and B). Stromal cells are virtually negative for staining. In colon tumour tissues, however, N-WASP protein staining was almost negative (Figure 2C and D).

Levels of N-WASP transcripts and the clinical and pathological links

Perhaps the most striking observation with levels of N-WASP in patients clinical samples is the significantly lower levels of N-WASP in tumour tissues compared with normal tissue (P<0.0001) (Figure 3A). Both node positive tumours and tumour with muscular invasion had significantly lower levels of N-WASP compared with node negative and non-invasive
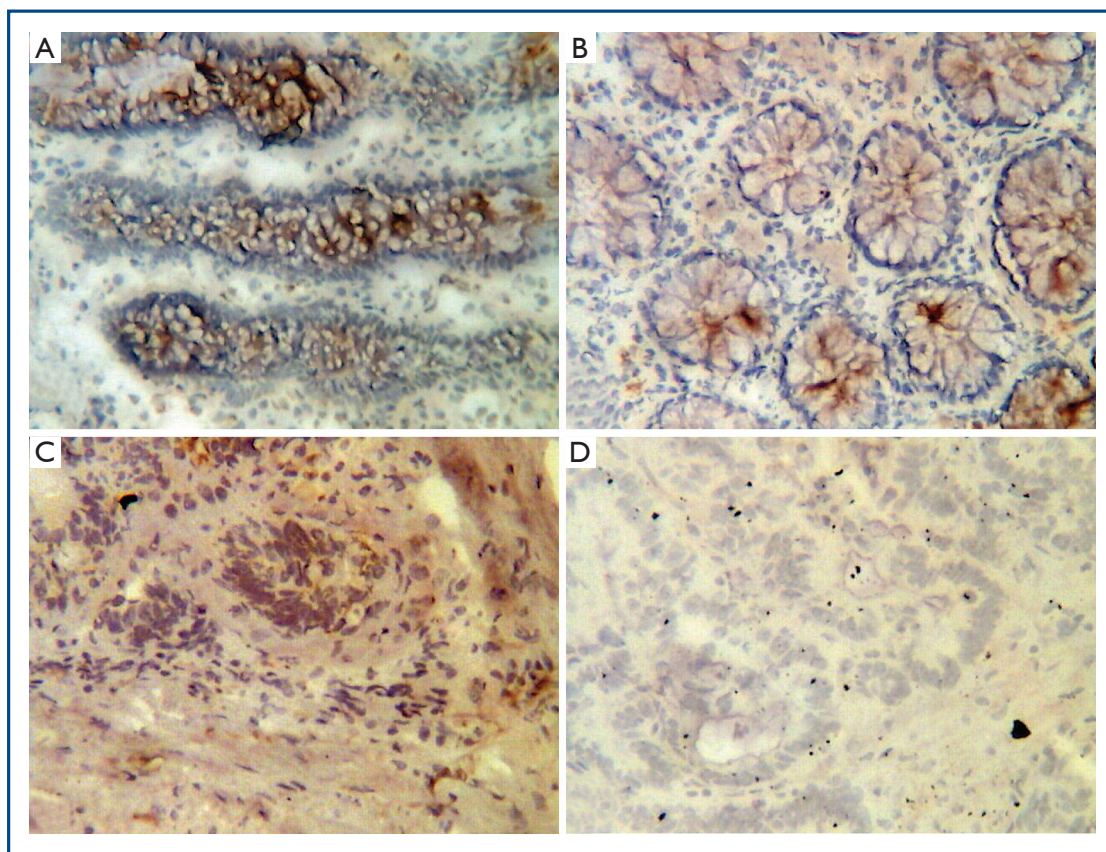
Figure 2. Immunohistochemical analysis of human N-WASP in human colon tissues. A and B: normal colon tissues; C and D: colon tumour tissues.

tumours (Figure 3D and F, respectively). When tumour staging is compared, there does not appear to be a clear link between N-WASP and TNM/Dukes staging. For example, TNM-2 tumours had higher levels of N-WASP than TNM1 and Dukes-B higher than Dukes-A tumours (Figure 3B/E). It is nonetheless interesting to observe that the most aggressive stages of the tumours, namely TNM3/4 and Dukes-C had lower levels than the moderate aggressive TNM-2 and Dukes-B tumours, although statistically this has yet to reach a difference. Finally, compared with patients who are alive, patients who died of colon cancer related causes had lower levels of N-WASP, although the difference is not statistically significant (P=0.068). Similarly, tumours from patients who developed distant metastasis also showed levels of N-WASP than tumours from those patients who remained disease free.

The relationship between N-WASP and long term survival

At the end of followup, patients were divided into those who were disease free, those who had developed distant metastasis

and those who died of colon rectal cancer (Table 1). Using the Kaplan-Meier survival model, we analysed the expression pattern and the long term survival of the patients. As shown in figure-4A, patients with low levels of N-WASP had a shorter overall survival [117 months (94-140) months] compared with those with higher levels [156 (131-181) months]. Similarly, low levels of N-WASP also associated with a shorter disease free survival [119 (98-140) months *vs.* 150 (122-177) months, for low and high levels, respectively] (Figure 4B).

Effect of N-WASP expression on the cellular function of colon cancer cells

Over-expression of N-WASP in HRT18 cells significantly reduced the rate of cell growth compared with control cells, although the effect of knocking down was less marked (Figure 5A). Interestingly, knocking down N-WASP significantly increased the adherence of the cells to extracellular matrix (Figure 5B). A significant opposite effect was seen when N-WASP was over-expressed. A similar pattern of relationship
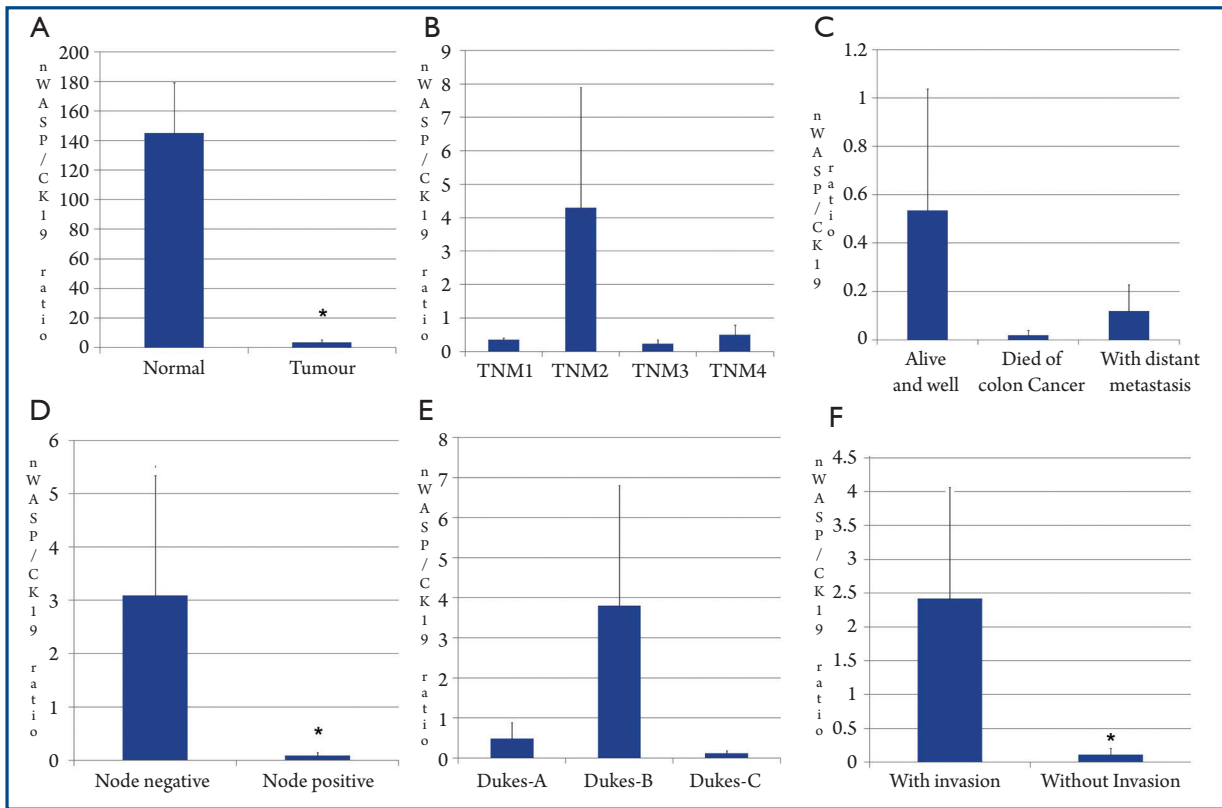
Figure 3. Expression of N-WASP transcript and the associated with clinical and pathological features.
A: comparison between normal and tumour tissues; B: N-WASP and TNM staging; C: N-WASP and clinical outcome; D: N-WASP and nodal status; E: N-WASP and Dukes staging; F: N-WASP and tissue invasion by tumour cells. Shown are N-WASP/CK19 ratio. *P<0.05.
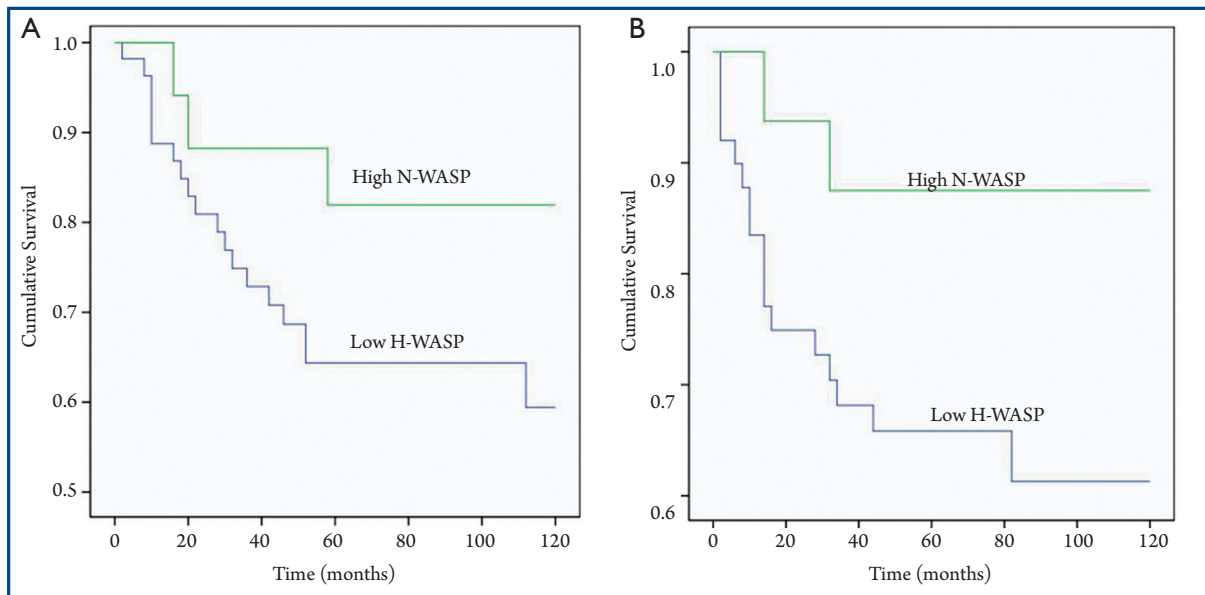
Figure 4. N-WASP expression and patients long term survival using Kaplan-Meier survival analysis. A: overall survival; B: disease free survival. Patients with low levels of N-WASP had short overall survival and disease free survival.

between N-WASP and cell motility (Figure 5C) and cell invasiveness (Figure 5D) was seen.

The potential involvement of the FAK pathway in N-WASP mediated cellular functions

In order to explore the potential role of the FAK in N-WASP mediated cellular functions, we exmployed a small specific FAK inhibitor in the cellular functions (Figure 6). The inhibitor had no marked effect on the growth and motility of the cells. However, it significantly reduced te invasiveness due to N-WASP knock down (Figure 6D).

Discussion

The present study has demonstrated that N-WASP, a potential cancer progression ? associated protein, had an aberrant expression pattern in human colon cancer. The study has shown that colon cancers had markedly lower levels of N-WASP at gene transcript and protein levels, as shown by both immunohistochemistry and quantitative PCR. The study further demonstrated that the levels of expression were associated with nodal status and muscular invasion and a low level was associated with a poor clinical outcome of the patients. This link appears to be reflected by the biological impact of N-WASP on colorectal cancer cells, in that levels of N-WASP were linked to the growth and invasiveness, possibly via the FAK pathway.

Colon tissues are highly positive for N-WASP and the N-WASP protein was largely seen in the cytoplasmic region of normal colonic epithelial cells. It is also interesting to observe that N-WASP protein staining was stronger in mature and differentiated epithelial cells compared with basal cells (Figure 2A/B). Tumour cells, from tissues and cell line alike, had much lower levels of N-WASP compared with normal cells. An interesting feature of the study on clinical samples is the inverse link between levels of N-WASP, lymph node involvement and tumour cell invasion of muscular layer. This inverse relationship is well reflected in the long term followup and patient's clinical outcome, namely low levels of N-WASP were seen in patients who died of colon cancer and the Kaplan-Meier model confirmed that these low levels were associated with shorter overall survival and disease free survival. Together, the data attempts to demonstrate a strong link between N-WASP and tumour invasiveness and clinical outcome. However, this link does not appear to be supported by the analysis on tumour staging (TNM and Dukes stagings). In that early tumours, namely TNM-1 and

Dukes-A appear to have lower levels of N-WASP compared with TNM-2 and Dukes-B tumours. Two possible reasons may have contributed to this observation, firstly the relatively smaller number of sample size in TNM-1 and Dukes-A groups; secondly, N-WASP may not be a good indicator for tumour staging. Clearly, a larger cohort would help to resolve this matter.

N-WASP belongs to a larger protein family, which include WASP, WAVEs and WISPs. Expression of N-WASP and the clinical implications of N-WASP have been reported in other tumour types. For example, in human breast cancer, N-WASP was reported to be expressed in a pattern similar to that reported here, namely reduced level of expression in aggressive tumours (16). In addition, other WASP family members have been studied in human colon cancer. WISP family has a different expression pattern from N-WASP. Aggressive colon tumours have markedly raised levels of WISP-1, whereas levels WISP-2 barely changed (24). An opposite trend was seen with breast cancer, in which WISP-1 was reduced in aggressive breast cancer cells and WISP-2 increased in the same tumours (25). Apart from the WASPs, the other members of the family have also been shown aberrant in human cancer. For example, WAVE-3 has been shown to connected to the disease progression and aggressive behaviour of prostate cancer and breast cancer (26,27). Again in human colorectal cancer, WAVE-2 has been indicated in the disease progression and clinical outcome of the patients (28).

To further understand the biological role of N-WASP in the behaviour of colon cancer cells, we created sublines from human colon cancer cell line, HRT-18, a cell weakly positive for N-WASP expression. Here, we created a subline in which N-WASP expression was knocked out and a subline N-WASP over-expressed. It was clearly demonstrated that over-expression of N-WASP markedly decreased the growth, adhesion and *in vitro* invasiveness of the cancer cells, with opposite effect seen when N-WASP was knocked down. The present study further revealed that inhibition of FAK by way of FAK inhibitor blocked increase in invasion due to N-WASP knockdown. It has shown previously that the FAK is able to affect the cellular location and activation of N-WASP in the cells thus the matrix adhesion of cells (29). It has also been reported that in breast cancer cells oestrogen is able to induce activation of FAK and the interaction between FAK and the N-WASP/ARP2/3 complex, which regulates cell migration (30). The present study is unable to provide further mechanism by which FAK and N-WASP interplay in colon cancer cells, but these recent studies provides vital information on the link between the two cellular protein complexes and will be useful
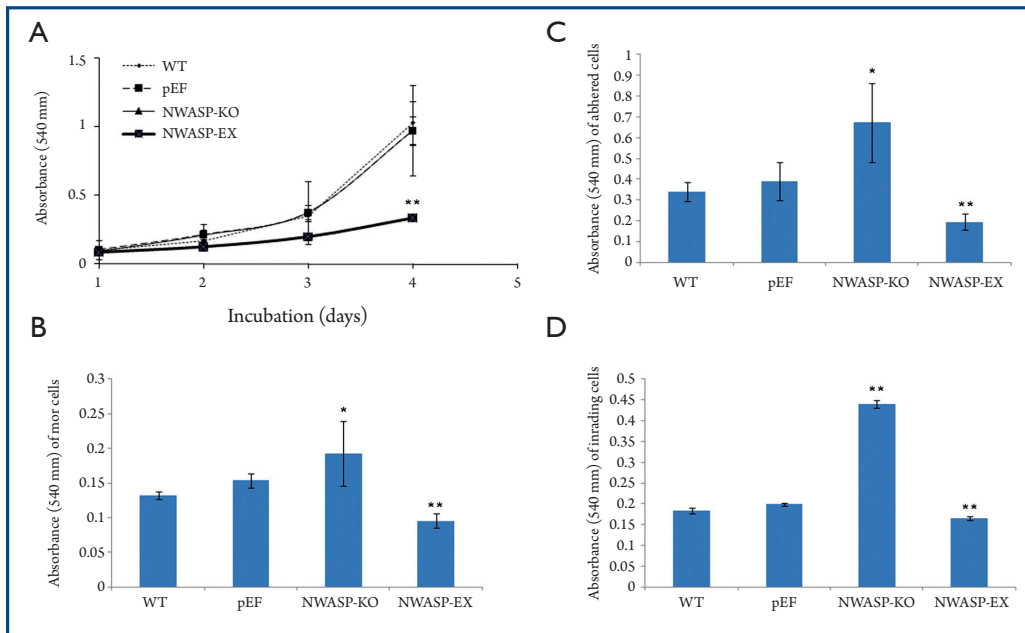
Figure 5. Expression of N-WASP and the biological function in colon cancer cells. A: Cell growth assay; B: Cell-matrix adhesion assay; C: cell motility assay and D: cell invasion assay. * and **: P<0.05 *vs.* wild type and control cells.
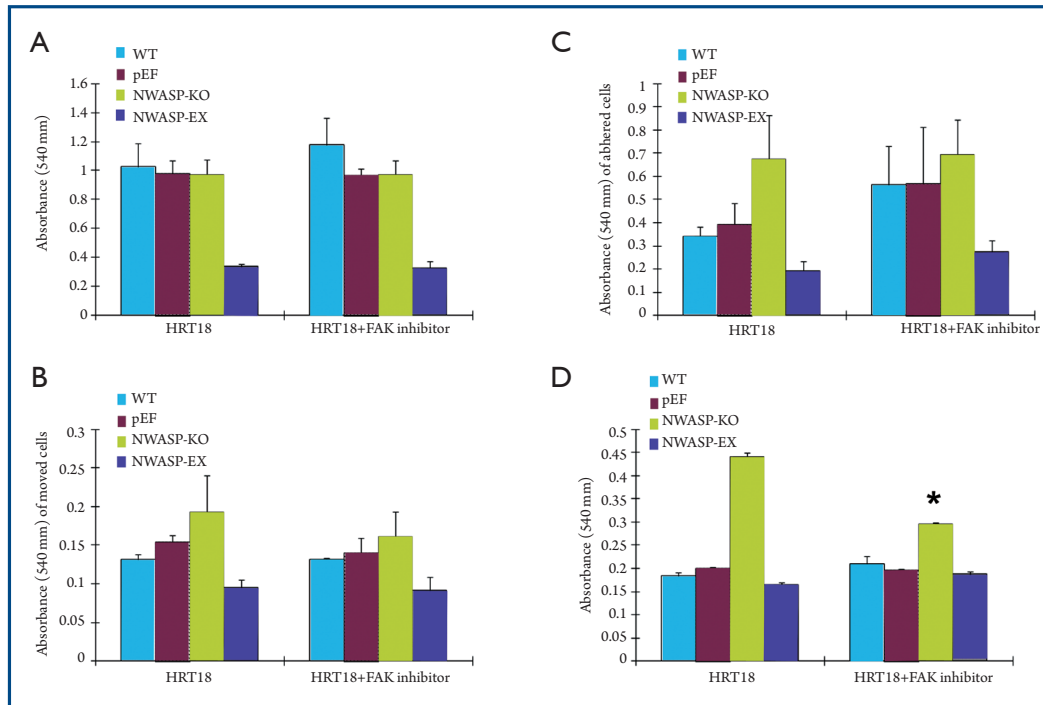
Figure 6. The potential role of FAK pathway in N-WASP mediated cell functions. A: Cell growth assay; B: Cell-matrix adhesion assay; C: cell motility assay and D: cell invasion assay. * P<0.05 *vs.* without FAK inhibitor.

future leads to explore, including mechanism beyond the FAK pathway (31).

In conclusion, N-WASP expression is aberrant in human colon cancer. A reduction of N-WASP in colon tumours is associated with disease progression and poor clinical outcome of the patients. In addition, N-WASP expression in colon cancer cells is inversely correlated with the aggressiveness of the cells, namely adhesion and invasiveness. Collectively, this study indicates that N-WASP carries the hallmark of a tumour metastasis suppressor in human colon cancer. This is likely to be via a FAK mediated pathway.

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