The traditional Tibetan medicine *Yukyung Karne* exhibits a potent anti-metastatic activity by inhibiting the epithelial to mesenchymal transition and cell migration

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Abstract

**Background:** In Traditional Tibetan medicine, *Yukyung Karne* has been used as the treatment of ovarian cancer. Though *Yukyung Karne* has been reported to be clinically effective, the molecular mechanism of its anti-metastatic action remains elusive.

**Methods:** The cytotoxic property of *Yukyung Karne* was evaluated by crystal violet staining while its ability to induce ceramide production was analyzed by sphingomyelinase assay. The anti-metastatic property was investigated using adhesion, invasion, migration and colony formation assays. The effect of *Yukyung Karne* on the expression of extracellular matrix components, and epithelial and mesenchymal markers, were evaluated by confocal microscopy and western blotting.

**Results:** *Yukyung Karne* exhibited a strong anti-metastatic property by significantly reducing the invasion, migration and colony formation ability of ovarian cancer cells. Besides it inhibited the levels of biomarkers involved in epithelial to mesenchymal transition such as down-regulation of vimentin and N-cadherin and up-regulation of epithelial E-cadherin. *Yukyung Karne* also induced the neutral sphingomyelinase II (nSMNaseII) enzyme activity that is known to hydrolyze sphingomyelins into pro-apoptotic intracellular molecule ceramide.

**Conclusions:** The study provides some compelling evidences supporting the anti-metastatic potential of *Yukyung Karne* which strongly suggests its possible usage as a promising alternative medicine. Thus, *Yukyung Karne* may be used as an anticancer and anti-metastatic agent along with other conventional anticancer therapeutics to increase their efficacy.

**Keywords:** *Yukyung Karne*, Traditional Tibetan medicine, Metastasis, Cell migration, Epithelial mesenchymal transition, Extracellular matrix

**Background**

Ovarian cancer is one of the leading gynecological malignancies worldwide in which tumor metastasis is associated with poor survival of the ovarian cancer patients [1, 2]. Tumor invasion and metastasis are recognized as complex and multi-step cellular processes which involve cell detachment, invasion, migration, intravasation, circulation, implantation, angiogenesis and proliferation [3]. Degradation of the extracellular matrix (ECM) by cancer cells through proteases such as matrix metalloproteinase’s (MMPs) and serine proteases aids to the separation of intercellular matrix and promotes tumor invasion. Incidentally, MMPs such as MMP2 (Gelatinase A) and MMP9 (Gelatinase B) are secreted by ovarian cancer cells which in turn correlates with increased occurrence of tumor invasion and metastasis. MMPs promote the epithelial to mesenchymal transition (EMT) by cleaving cell adhesion molecule E-cadherin [4]. Therefore, interventional strategies against these steps are considered important to prevent metastasis [5, 6]. Further, loss or
reduction of trans membrane glycoprotein E-cadherin is another important manifestation in the EMT program of tumor cells. It is now well established that loss of E-cadherin and up-regulation of mesenchymal markers result in subsequent activation of signal transduction events such as Wnt/β-catenin pathway that aid to the progression of metastasis [7]. Not surprisingly, the levels of Wnt effector β-catenin are tightly regulated in order to control the expression of its target genes such as c-myc that exerts a profound effect on cell proliferation, differentiation and migration [8].

Chemotherapy, surgery and radiotherapy are widely used in the management of cancer but these are often associated with adverse effects. To remove these short-comings in cancer treatment, much attention has shifted towards the use of complementary and alternative medicines (CAM). Not surprisingly, CAM is now fast emerging as an effective measure to aid conventional therapies [9]. Natural products have been widely used for centuries and recent preclinical studies have shown their potential applications in pharmacology and cancer therapy. Yukyung Karne has been used for many decades in traditional Tibetan medicine or Sowa Rigpa for treatment of cancer [10]. Recently, we have reported the anticancer potential of Yukyung Karne using ovarian cancer cells in culture [11]. In the present study, we have investigated the anti-metastatic properties of Yukyung Karne on ovarian cancer cells by evaluating its inhibitory effects on cell migration, cell invasion, EMT and ECM.

Methods

Cell lines and culture conditions

Ovarian cancer cell line SKOV6 was a kind gift of Dr. Anil Suri (National Institute of Immunology, New Delhi). All cultures were grown in Dulbecco’s modified Eagle’s medium containing 10% heat inactivated fetal bovine serum, penicillin 100 μg/ml and streptomycin (100 μg/ml). Cells were incubated at 37 °C in a humidified chamber under 5% CO₂ atmosphere.

Traditional Tibetan medicine

Yukyung Karne was purchased from the Tibetan Medical and Astrological Institute, Dharamshala, India. According to the Tibetan Pharmacopoeia [10]. Yukyung Karne is a mixture of various plant components including: Roots of Saussurea lappa (C.B. Clarke) (family: Asteraceae), Zingiber officinalis (Roscoe) (family: Zingiberaceae), Aconitum ferox (WallLex Ser) (family); Corydalis hendersoni (Fedde) (family: Fumariaceae), and Acorus calamus (L) (family: Araceae); Fruits of Emblica officinalis (L) (family: Euphorbiaceae), Piper longum (L) (family: Piperaceae), and Terminalia chebula (Retz) (family: Combretaceae); Leaves of Adhatoda vasica (NEES) (family: Acanthaceae); Seeds of Ellettra cardomomum (L) (family: Zingiberaceae), Coriandrum sativum (L) (family: Apiaceae), Embelia ribes (Burn, F) (Family: Myrsinaceae), and Delphinium brunonianum (Royale) (family: Ranunculaceae); resin of Commiphora mukul (Hook) (family: Burseraceae); Whole plant of Meconopsis horridula (Hook) (Papaveraceae), and Dracocephalum tanguticum (Maxim) (family: Lamiaceae); and Tsbool (detoxified mercury) as mineral ingredient. All the herbs were identified by Dr. Tsering Norbu (Menrampa) and the voucher numbers of plant specimens are available at the herbarium department of Tibetan Medical and Astrological Institute for reference.

Sphingomyelinase activity

Neutral sphingomyelinase (nSMase II) enzyme activity was determined in cell lysate using Amplex Red Sphingomyelinase Assay Kit (Molecular probes, Invitrogen) as per manufacturer’s instructions. The reaction was carried out for 30 min and fluorescence data was measured in a fluorescence microplate reader using excitation at 530 nm and emission 590 nm. The fluorescence values were normalized with total protein to obtain activity/μg of protein.

Gelatin zymography

Gelatin zymography was performed as described by Al Dhaheri et al. [12]. Briefly, cells were incubated in serum free DMEM for 24 h in the presence of Paclitaxel (P), Yukyung Karne (YK) or P + YK (PY). The conditioned media was collected and protein was precipitated (1:4) by acetone. Samples were resuspended in 2x Laemmli buffer without reducing agent. After boiling, the samples were resolved in a 10% poly acrylamide gel containing 0.1% gelatin. After electrophoresis, gels were washed with 2.5% Triton X100 at RT to remove SDS and then incubated overnight at 37 °C with substrate incubation buffer (50 mM Tris–HCL pH 7.5, 200 mM NaCl, 10 mM CaCl₂). Bands corresponding to MMP2/9 activity were visualized by negative staining using Coomassie brilliant blue. Images were captured using Fluorchem M (Protein Simple, USA).

Crystal violet staining of cells

SKOV6 cells were treated with Yukyung Karne. After 24 h, media was discarded and adhered cells were washed with 1xPBS and fixed in methanol. Crystal violet (1% solution) was added to the cells for 20 min followed by a wash, and the stained cells were resuspended in methanol and absorbance was read at 600 nm.

Cell adhesion assay

Cover slips were coated with collagen type I at 37 °C for 1 h. SKOV6 cells were seeded in the presence of Paclitaxel (P), Yukyung Karne (YK) or P + YK (PY). After16–20 h incubation at 37 °C in a humidified 5% CO₂ incubator, unadhered cells were gently removed by washing the coverslips with 1x PBS. Subsequently, the adhered cells
were fixed in ice cold methanol: acetone (1:1) for 10 min at −20 °C. Cells were stained with Hoechst dye for 15 min at RT. After rinsing with 1x PBS, images were captured using confocal microscopy (Nikon A1R). Adherent cells were counted in five random fields, and the mean number of cells/field ± SD was calculated.

**Colony formation assay**
The colony formation assay was performed as described by Shukla et al., [13]. Briefly, treated cells were incubated at 37 °C for 14 days and observed regularly for colony formation and the bright field images were captured with a Nikon ECLIPSE TE 2000-S inverted microscope and the number of foci were counted in random fields.

**In-vitro invasion assay**
The cell migration of SKOV6 cells was studied using Trans-well plate with 8 μm pore size membrane insert. Complete medium was added to the membrane and lower chamber 1 h prior to seeding of cells at 37 °C, 5 % CO₂ humidified incubator. Cells were seeded to the membrane and treatment was simultaneously given for 4 h. After incubation, inserts were carefully removed and cells were scraped off using cotton swabs from the upper surface of the membrane. Cells on lower side of the membrane were fixed in 10 % methanol followed by staining with crystal violet (1 %) for 20 min. The membranes were air dried and the number of infiltrated cells on the lower side of filter was counted by microscopy.

**Wound healing assay**
Briefly, SKOV6 cells were grown as monolayer at 37 °C, 5 % CO₂ in a humidified incubator. A scratch wound was made using 200 μl yellow pipette across the confluent monolayer. Cells were cultured with fresh DMEM supplemented with 10 % fetal bovine serum in the presence or absence of Yukyung Karne (100 μg) for 24 h. The extent of wound closure was captured using Nikon ECLIPSE TE 2000-S inverted microscope.

**RNA extraction and quantitative real time PCR (RT-qPCR)**
Total RNA was isolated from cells using Trizol reagent (Invitrogen) as per the manufacturer’s protocol. Reverse Transcriptase-PCR (RT-PCR) was performed with MMuLV reverse transcriptase (Fermentas) according to the manufacturer’s protocol. qRT-PCR was done in an ABI Step One plus system (Applied Bio system) using specific primers for N-Vimentin Forward: 5’GAGACAGGTGCAGTCCCTCA, Vimentin Reverse: 5’GAAAGTGACGAGCCATTTCC. β Actin served as an internal control.

**Western blotting**
Cell lysates were prepared in 1x cell lysis buffer (Promega, USA). Protein concentration was determined by Bradford’s reagent (Biorad). Equal amounts of protein samples were resolved in a 10-15 % SDS-PAGE followed by western blotting (WB). Primary antibodies-E-cadherin, N-cadherin were procured from Cell Signaling Technology, pFAK (Y397), Caveolin1 (N-20), Integrin5α, Beta catenin (E5), GSK-3β (H-76) and GAPDH (FL-335) were from Santa Cruz Biotechnology (USA). The protein bands were visualized using enhanced chemiluminescence (ECL) from Santa Cruz Biotechnology (California, USA).

**Statistical analysis**
Data are expressed as mean ± S.D. Statistical significance was calculated using Student’s t test. p values of <0.05 were considered significant.

**Results**
Yukyung Karne elevates the nSMNase II activity
Working on the pharmacological basis of anticancer properties of Yukyung Karne, we have earlier reported that it is able to induce cell cycle arrest and apoptosis as well as restore the levels of the key tumor suppressor factor p53 in
ovarian cancer cell line SKOV6. Since manipulation of sphingolipid levels for ceramide production has been shown to improve the efficacy of anticancer therapy [14, 15], we investigated the effect of *Yukyung Karne* on the activity of nSMaseII enzyme in SKOV6 cells. Interestingly, we observed the elevated levels of nSMaseII in SKOV6 cells treated with YK (100 μg) (*p* <0.001) confirming its positive role in ceramide production (Fig. 1a). We also observed that *Yukyung Karne* treatment significantly inhibited the viability of the cells (*p* <0.001) as evident from the crystal violet staining of cells (Fig. 1b) further strengthening its pro-apoptotic property.

**Yukyung Karne inhibits collagen-mediated cell adhesion**

Since the migratory and metastatic potential of cancer cells is fore timed by their interaction with endothelial cells and ECM proteins like collagen [16], we assessed the effect of *Yukyung Karne* treatment on cell to matrix interaction *in vitro* by collagen binding ability using ovarian cancer cells. According to our data, a quantum reduction in adhesion of cancer cells to collagen (84 %) was observed for *Yukyung Karne* treated SKOV6 cells when compared to untreated cells. Interestingly, a significant decrease in cell adhesion was also observed for positive control paclitaxel (*p* <0.05) and combination of paclitaxel with YK (*p* <0.001) suggesting its strong ability of *Yukyung Karne* to suppress the adhesion of SKOV6 cells to collagen (Fig. 2a).

**Yukyung Karne interferes with the transformation of SKOV6 cells**

Widely accepted hallmarks of cancer include the resistance of cancer cells to contact inhibition and the enhanced ability to form independent colonies. We next examined the inhibitory potential of *Yukyung Karne* on the colony forming ability of SKOV6 cells. While the untreated SKOV6 cells produced 15 ± 3 colonies/field, it was suppressed to 5 ± 1 (*p* <0.001) on exposure to YK, Pac 7.5 ± 1 (*p* <0.001) and PY 5.5 ± 1 (*p* <0.001) (Fig 2b). This result confirmed the negative effect of *Yukyung Karne* on the transformation potential of ovarian cancer cells.

**Yukyung Karne inhibits migration and invasion of ovarian cancer cells**

Metastasis of ovarian cancer cells from the primary site to the neighboring secondary tissues mainly peritoneum and omentum, the main event in transition of benign to malignant cancer. To elucidate the role of *Yukyung Karne* in inhibiting the migratory potential of SKOV6 cells, we performed trans-well migration and wound healing assays. A close examination of the invasive potential of *Yukyung Karne* using transwell insert (Fig. 3a) revealed a significant reduction in the number of infiltrated cells in a dose

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**Fig. 2** Inhibition of cell adhesion to collagen and colony formation in the presence of *Yukyung Karne*. **a** SKOV6 cells were grown in presence of Paclitaxel (Pac, 10 nM), *Yukyung Karne* (YK, 100 μg) or PY [Pac (10 nM) + YK (100 μg)] and allowed to adhere to collagen-coated (10 μg/ml) cover slips for 24 h. The adhered cells were quantified using Hoechst dye. **b** Cells were treated as above and grown in soft agar for 14 days and the number of colonies was counted. All results represented here are mean ± S.D. from three independent experiments. Statistical significance: *, *p* <0.05; **, *p* <0.001.
Fig. 3 (See legend on next page.)
dependent manner (p < 0.001). Further, in wound healing assay (Fig. 3b), Yukyung Karne severely impaired the migratory behavior of SKOV6 cells when compared to untreated cells by 20 % and 80 % at 6 h and 24 h respectively. Taken together these results confirm the inhibitory effect of Yukyung Karne on the migration and invasion potential of SKOV6 cells.

Yukyung Karne acts as a MMPs inhibitor

Degradation of the ECM by MMPs aids in tumor invasion and thus is a crucial step in the initiation of metastasis [17]. In line with its strong anticancer properties, we next investigated the inhibitory effect of Yukyung Karne on both MMP2 and MMP9 activities using gelatin zymography. As shown in Fig. 4a, there was a marked inhibition (p < 0.05) in the gelatinolytic band corresponding to MMP9 and MMP2 (proenzyme forms) in the conditioned media of the cells treated with Yukyung Karne when compared to control untreated cells.

Effect of Yukyung Karne on EMT

In order to metastasize, tumor cell undergo an EMT that is marked by increase in mesenchymal markers such as vimentin, vitronectin, N-cadherin and decrease in epithelial markers like E-Cadherin [18]. Since Yukyung Karne caused down regulation of MMPs we hypothesized that it may have a strong bearing on the suppression of EMT markers. Western blot analysis of the Yukyung Karne treated cells showed a marked inhibition of N-cadherin protein (p < 0.05) (Fig. 4b) and a significant inhibition (p < 0.01) in the levels of vimentin mRNA in the Yukyung Karne treated cells (Fig. 4c). In contrast, as shown in Fig. 4b, the levels of E-cadherin were found to be significantly elevated following treatment with PY (p < 0.01) and YK (p < 0.05). Thus, the reciprocal expression of epithelial and mesenchymal markers induced by Yukyung Karne, suggest its potential as an anti-metastatic agent.

Effect of Yukyung Karne on ECM

To further support the anti-metastatic property of Yukyung Karne, we also investigated its role in the modulation of Wnt/β-catenin pathway. Binding of Wnt protein to the receptors triggers activation of β-catenin and its inappropriate activation was often found deregulated in many cancers. Under normal circumstances oncoprotein β-catenin gets phosphorylated by GSK3β and subsequently ubiquitinylated and targeted for degradation. Western blot analysis of the Yukyung Karne-treated SKOV6 cells exhibited a significant reduction in the Wnt/β-catenin effector molecule β-Catenin (p < 0.05), GSK3β (p < 0.05), and also its downstream target c-Myc (p < 0.05), (Fig. 5c) [19, 20]. Consistent with a marked inhibition of ECM components and its cytoskeletal dynamics, the levels of integrin-5 which is crucial for cell to cell and ECM interaction for invasion was down-regulated following Yukyung Karne treatment (p < 0.001) (Fig. 5c).

To further understand the mechanism of action of Yukyung Karne, we investigated its effect on the levels of Caveolin-1 whose down regulation is associated with activation of Wnt/β-catenin kinase and anchorage independent growth [21]. Surprisingly, the Yukyung Karne-treated cells exhibited no appreciable change in the levels of Caveolin-1 (Fig. 5a, c) while there was a marginal (40 %) inhibition in the levels of pFAK which is crucial for transmitting signals during tumorgenesis (Fig. 5b, c). Taken together, our present sets of results strongly suggest the inherent potential of Yukyung Karne. Yukyung Karne exerts an inhibitory effect on the migration of ovarian cancer cells by regulating the expression of EMT markers and ECM components.

Discussion

CAMs are fast emerging as an acceptable choice for treating various chronic diseases including ovarian cancer where extensive metastasis to the neighboring organs like fallopian tubes, uterus, bladder and peritoneal cavity is a major challenge. In traditional Tibetan medicine, Yukyung Karne has been used as an effective alternative treatment for cancer metastasis specifically for ovarian cancer. Recently, we have shown that Yukyung Karne exhibits several therapeutic effects on ovarian cancer cells such as induction of growth arrest and apoptosis [11]. In addition to our earlier observation, we observed a marked increase in the levels of a critical enzyme nSMaseII which is involved in generation of ceramide from sphingomyelins in response to apoptotic inducers such as chemotherapeutic agents leading to growth inhibition and apoptosis [14]. Thus, increase in the nSMaseII activity appears to be one of the Yukyung Karne's cytotoxic mechanisms. However, the pharmacological basis of its anti-metastatic property remains elusive. In the present study, we investigated the anti-metastatic potential of Yukyung
Karne using ovarian cancer cells. Adhesion of cells to ECM is a crucial event in modulation of cellular processes including the regulation of anchorage-dependent processes. Interestingly, a significant number of cancer cells failed to adhere to the collagen-coated culture plates in the presence of Yukyung Karne (Fig. 2). Further, cell migration is an important mechanism in the metastasis of high-grade ovarian cancer [22]. We observed a negative correlation between the number of migratory cells and the dose of Yukyung Karne. Further, the colony forming ability
of SKOV6 cells were also seen reduced significantly upon treatment with Yukyung Karne.

Here EMT plays a major role in aiding the tumor cells to migrate out from the primary site, enter into the circulation and adhere to endothelial cells of target organs. Cadherin-mediated adhesion plays an important role in maintaining cell-cell contacts and reducing tumor metastasis [23, 24]. Accordingly, the E-cadherin negative tumor is a predictor of poor overall survival [25]. In the present study, we observed a striking correlation in the E-cadherin level and decrease in mesenchymal markers such as Vimentin and N-cadherin supporting epithelial characteristics following treatment with Yukyung Karne and correlates with decrease in tumorogenicity [26].

Degradation of ECM components is a prerequisite step for initiation of metastasis [27] and intraperitoneal ovarian cancer metastasis is mediated by adhesion via integrins to peritoneal mesothelial cells. On binding to ECM, integrin 5α regulates diverse functions in tumor cells including migration, invasion, proliferation and survival through activation of various pathways such as MAPK.

In our study, we observed a significant decrease in integrin 5α and pFAK gene expression corroborating the tumor growth inhibitory property of Yukyung Karne (Fig. 5b, c). E-cadherin is frequently down regulated in epithelial tumors and a number of studies have shown that disruption of E-cadherin leads to transcriptional activation of oncoprotein β-catenin [23]. The accumulation of β-catenin stimulated transcription factors that enhanced cell proliferation and poor prognosis. Our study confirmed decreased levels of β-catenin, pGSK3-β and downstream target c-Myc (Fig. 5c) and increased levels of E-cadherin (Fig. 4b) following Yukyung Karne treatment.

Besides the well-defined role of major ECM components, we also investigated the status of scaffolding protein Caveolin in this study. Caveolin 1 is ubiquitously expressed and encodes a major component of membrane caveolae. It functions as tumor suppressor [31] and contributes to the organization and stability of adherent junctions through its association with E-cadherin at junction level. Caveolin 1 is consistently seen lost or down regulated in malignant ovarian cancers [23]. Interestingly, the level of caveolin1 levels were found up-regulated in the Yukyung Karne treated SKOV6 cells suggesting Yukyung Karne as a strong candidate in inducing expression of tumor suppressor caveolin 1 (Fig. 5c). Consistent with anti-metastatic property of Yukyung Karne, further our gelatin zymography studies also indicated a marked reduction in the activity of key enzymes MMP2/9 (Fig. 4a) that are involved in the cleavage of

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**Fig. 5** Effect of Yukyung Karne on extracellular matrix proteins. SKOV6 cells were cultured on cover slips and treated with Paclitaxel (Pac, 10 nM), Yukyung Karne (YK, 100 μg) or PY (Pac (10 nM) + YK (100 μg)) for 24 h and processed for immunofluorescence analysis using anti caveolin 1 antibody (a), and with pFAK antibody (b). c Cell extracts were western blotted (WB) to show the levels of β Catenin, pFAK, pGSK3β, c-Myc, Caveolin 1 and Integrin. Actin was used as loading control. Similar results are obtained from three independent experiments.
ECM components during tumor invasion and metastasis and are abundantly expressed in various malignant cancers [32, 33].

Thus, Yukyung Karne seems to exert its anti-metastatic potential by regulating EMT with increase in E-cadherin expression and loss of critical intracellular oncoprotein β-catenin that has been shown implicated in induction of EMT in various cancers [7]. However, further in vivo studies are needed to ascertain its effectiveness in the treatment of ovarian cancer. Nevertheless, Yukyung Karne appears to be a good candidate medicine for treating ovarian cancer patients as it exhibits better efficacy than paclitaxel. Further, its use in combination therapy along with conventional chemotherapeutic agent such as paclitaxel should improve the overall efficacy of treatment.

Conclusions
Our findings provide a multitude of evidences supporting a strong and potent anti-metastatic property associated TTM Yukyung Karne. Yukyung Karne effectively counters a range of anti-metastatic properties of cancer cells including adhesion, invasion migration, colony formation, ECM components, loss of mesenchymal marker and gain of epithelial marker. Thus, Yukyung Karne appears to be an ideal CAM that could be used for developing new therapeutic strategies in the management of ovarian cancer metastasis.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
TC and GM carried out experiments and drafted the manuscript. VK and GM carried out experiments and drafted the manuscript. Authors have read and approved the final manuscript.

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