Mechanistic insights into the therapeutic potential of the Traditional Tibetan Medicine-*Yukyung Karne* in ovarian cancer cells

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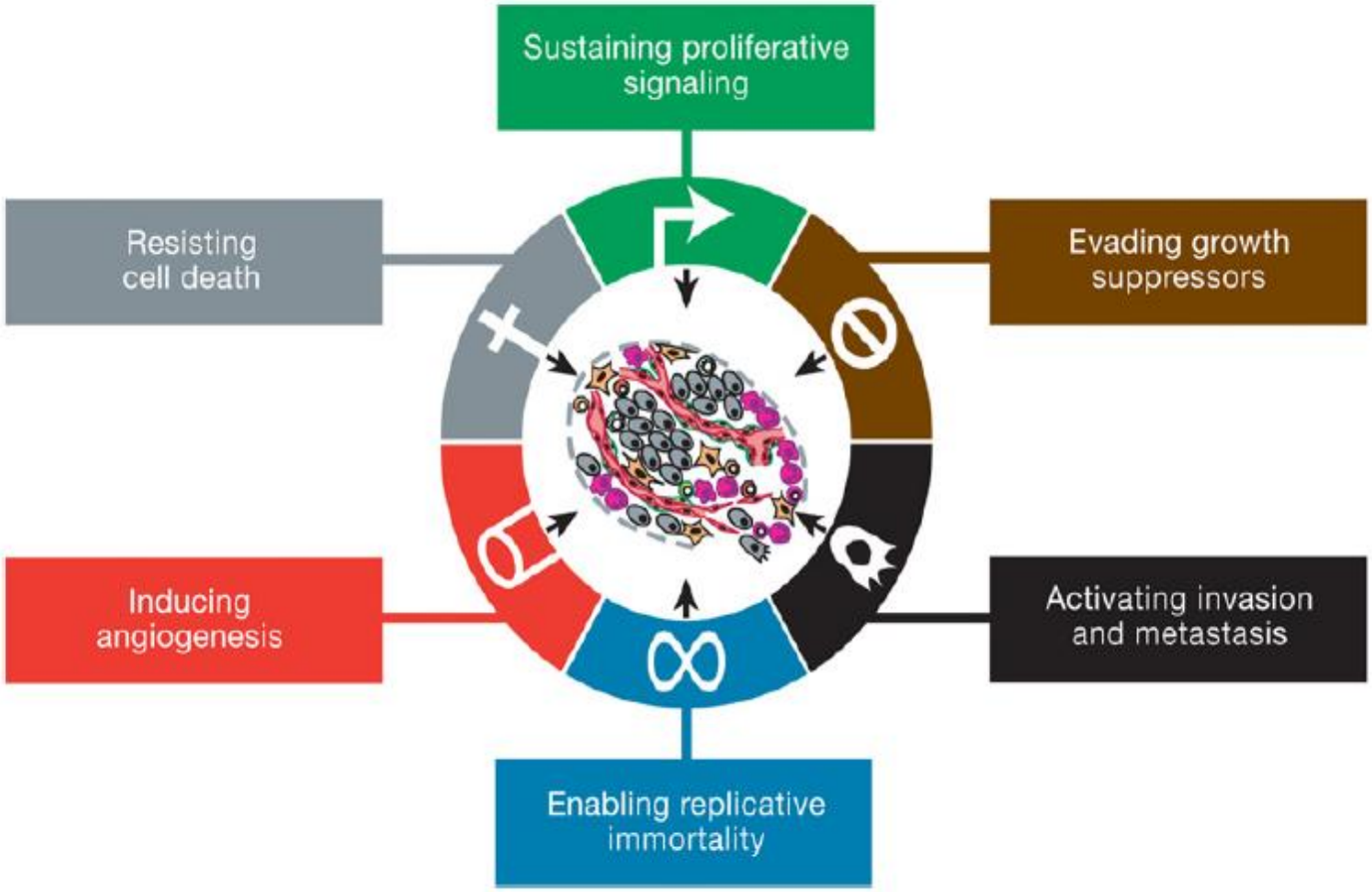
Hallmarks of cancer

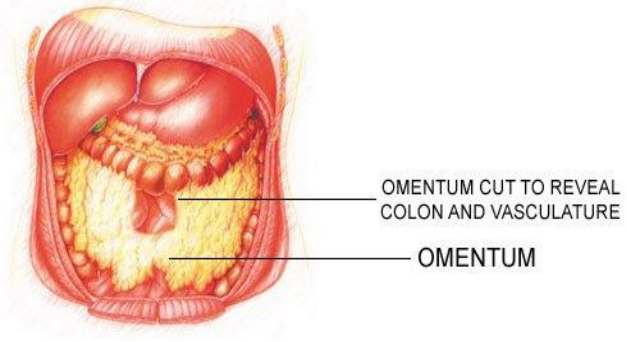


Hanahan and Weinberg, Cell, 144, 2011

Ovarian cancer

- Ovarian cancer is the sixth leading cause of cancer in women and seventh the leading cause of cancer death globally.
- Difficult to diagnose
- Late diagnosis: Stage 3 cancer of the ovary
- 80% of Ovarian cancer present with omental metastasis
- Screening test: CA 125 (50%) accuracy, late marker
- Standard treatment : no change in survival rate

Scenario in India



✓ The projected number of cases for this cancer in India for 2015 and 2020 are 45,231 and 59,276, respectively

Risk Factors Associated with Ovarian Cancer

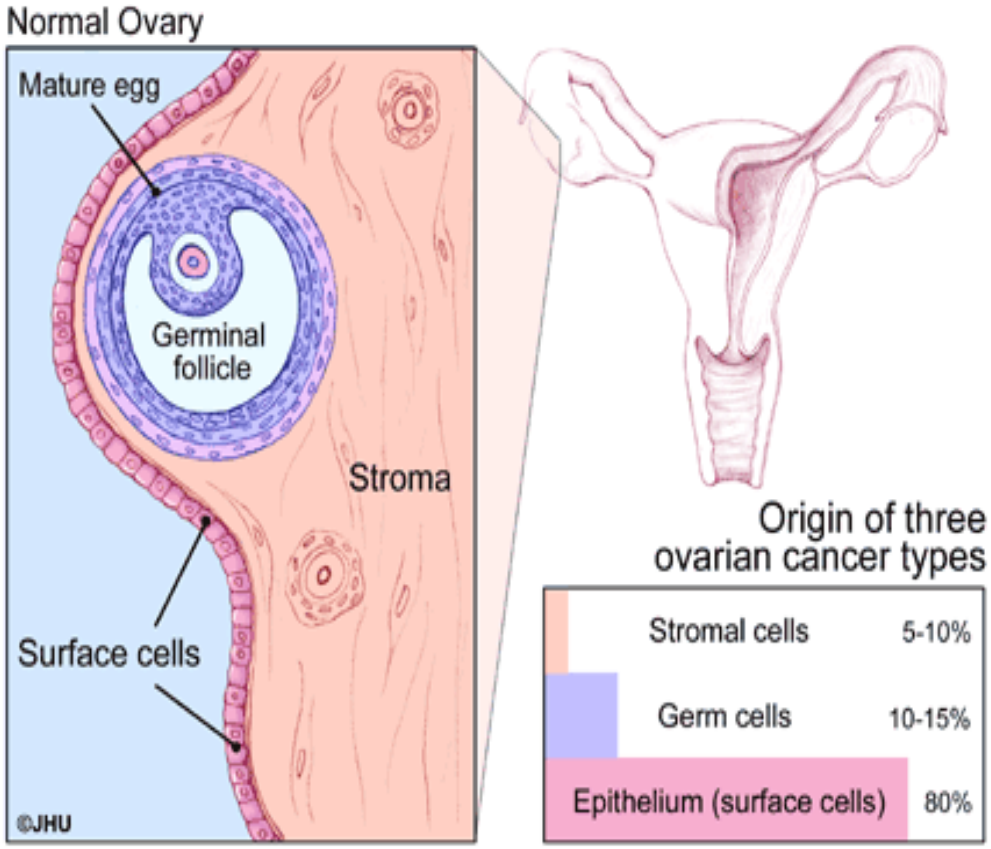
Increased Risk

- Delayed childbearing
- Early menarche
- Endometriosis
- Estrogen replacement therapy for more than five years
- Family History suggesting genetic predisposition
- Genetic syndromes
- Hight fat diet
- Late menopause
- Low parity

Decreased Risk

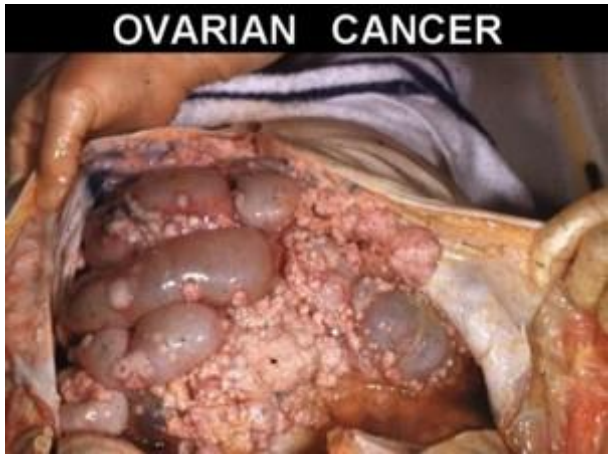
- Breastfeeding for 18 months or more
- Early menopause
- Multiparity (risk decreases with each additional pregnacy)
- Hysterectomy
- Late menarche
- Low fat diet
- Tubal Ligation

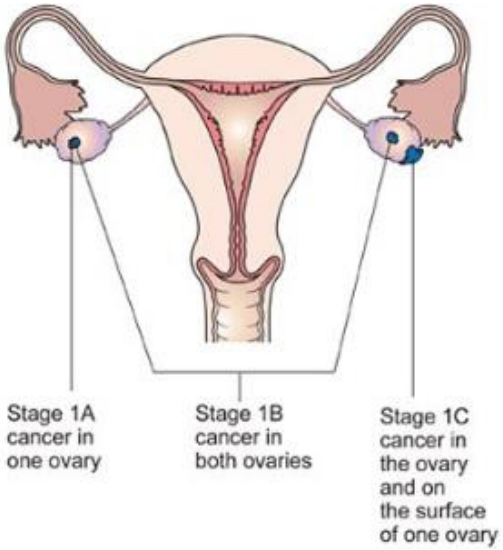
Types of ovarian cancer

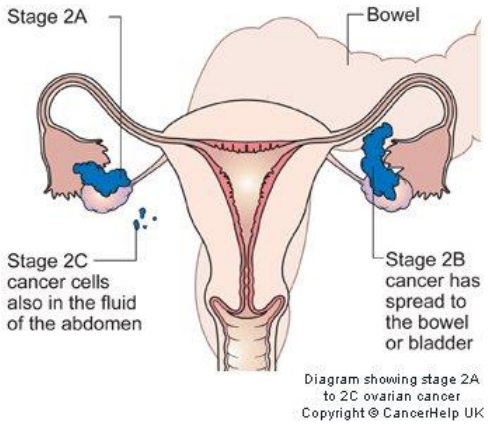


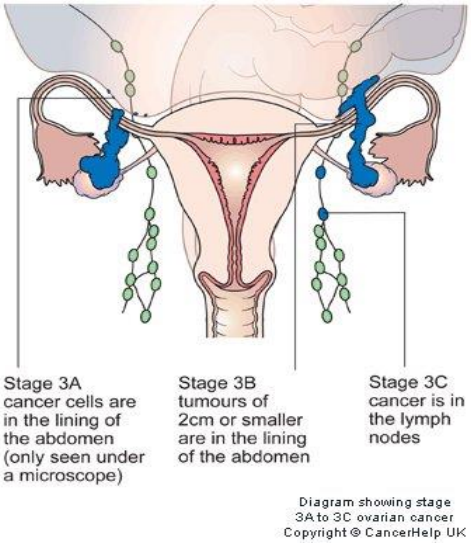
1. Epithelial ovarian cancer
2. Germ cell ovarian cancer
3. Stromal ovarian cancer

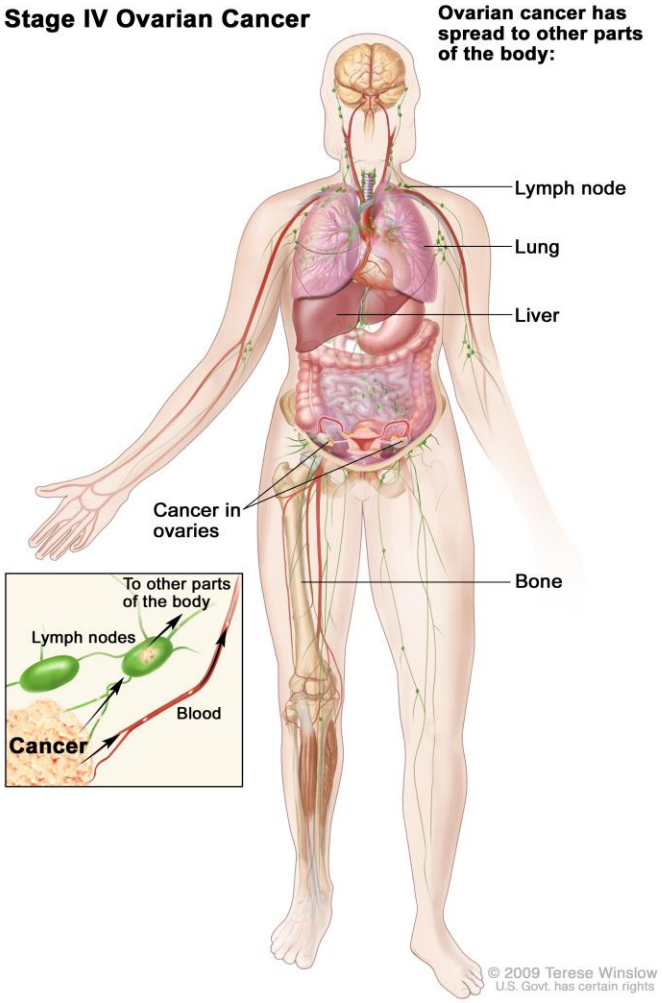
Different stages of ovarian cancer











Standard treatment of Ovarian cancer

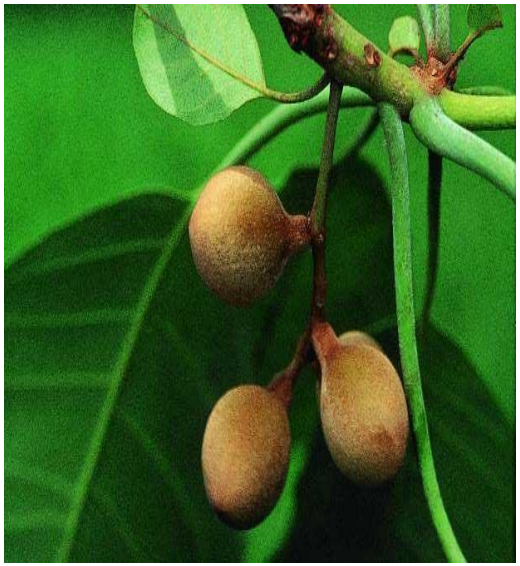
- ✓ Surgery , Radiotherapy and chemotherapy to kill the cells that are dividing uncontrollably.
- ✓ Chemotherapy : Platinum based drugs - paclitaxel and carboplatin - are the drugs most widely used. Tumours usually respond to this form of treatment but often eventually return.
- ✓ ONX-0801 is the first in a brand new class of drugs, discovered at the ICR, which attacks ovarian cancer by mimicking folic acid to enter cancer cells. The drug kills cells by blocking a molecule called thymidylate synthase and causing irreparable DNA damage

Traditional Tibetan Medicine (TTM)

- Century old medical system of holistic and naturopathic approach
- The root of TTM integration of the major medical system of the ancient world (India, China, Persia and Greek)
- Formulations are multi components of herbs and minerals
- Known to cure the root causes of diseases
- Effective against chronic diseases like cancer
- Dosage of individual components are very low

Three precious jewels of Tibetan Medicine (Aru Baru kyuru 2:1:1)

Terminalia chebula (Aru ra)



Terminalia belerica (baru ra)

Phyllanthus emblica (kyuru ra)

Yukyung Karne

Multi herbal components of Yukyung Karne

Root of ***Saussurea lappa*** (C.B. Clarke) (family: Asteraceae)
Fruit of ***Emblica Officinalis*** (L) (family: Euphorbiaceae)
Leaves of ***Adhatoda vasica*** (NEES) (family: Acanthaceae)
Seeds of ***Elletaria cardomomum*** (L) (family: Zingiberaceae)
Fruit of ***Piper longum*** (L) (family: Piperaceae)
Whole plant part of ***Dracocephyllum tanguticum*** (Maxim) (family: Lamiaceae)
Root of ***Zingiber officinalis*** (Roscoe) (family: Zingiberaceae)
Seed of ***Coriandrum sativum*** (L) (family: Apiaceae)
Whole plant of ***Meconopsis horridula*** *(Hook)* (Papaveraceae)
Root of ***Corydalis hendersoni*** (Fedde) (family: Fumariaceae)
Seeds of ***Embelia ribes*** (Burm. F) (Family:Myrsinaceae)
Delphinium brunonianum (Royale) (family: Ranunculaceae)
Fruit of ***Terminalia chebula*** (Rety) (family: Combretaceae)
Root of ***Acorus calamus*** *(*L) (family: Araceae)
Root of ***Aconitum ferox*** (Wall.ex Ser) (family: Ranunculaceae)
Resin of ***Commiphora mukul*** (Hook) (family: Burseraceae)

Multi herbal components of *Yukyung Karne*

Sassurea lappa

Zingiber officianalis

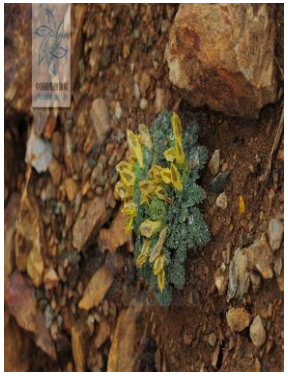
Adhatoda vasica

Acorus calamus

Aconitum ferox

Commiphora mukul

Coriandrum sativum



Corydalis hendersoni

Delphinium brunonianum

Dracocephyllum tancuticum

Embelia ribes

Emblica officianalis

Elletaria cardamomum

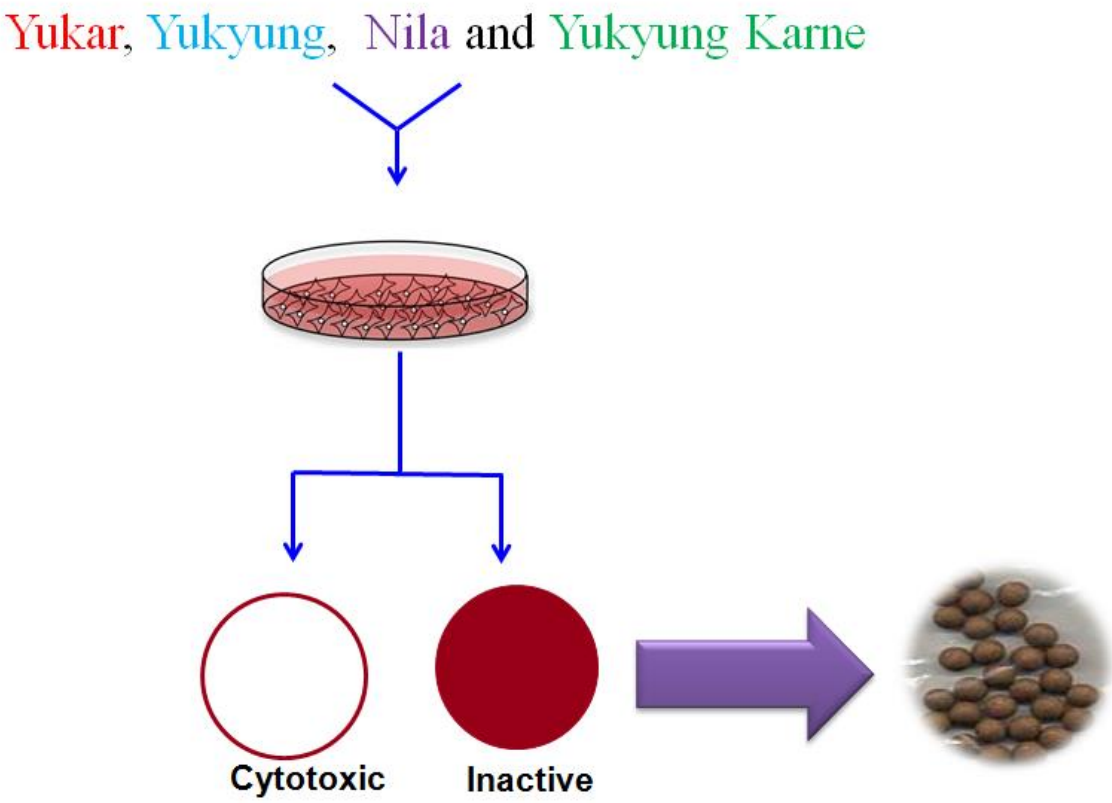
Meconopsis horridula

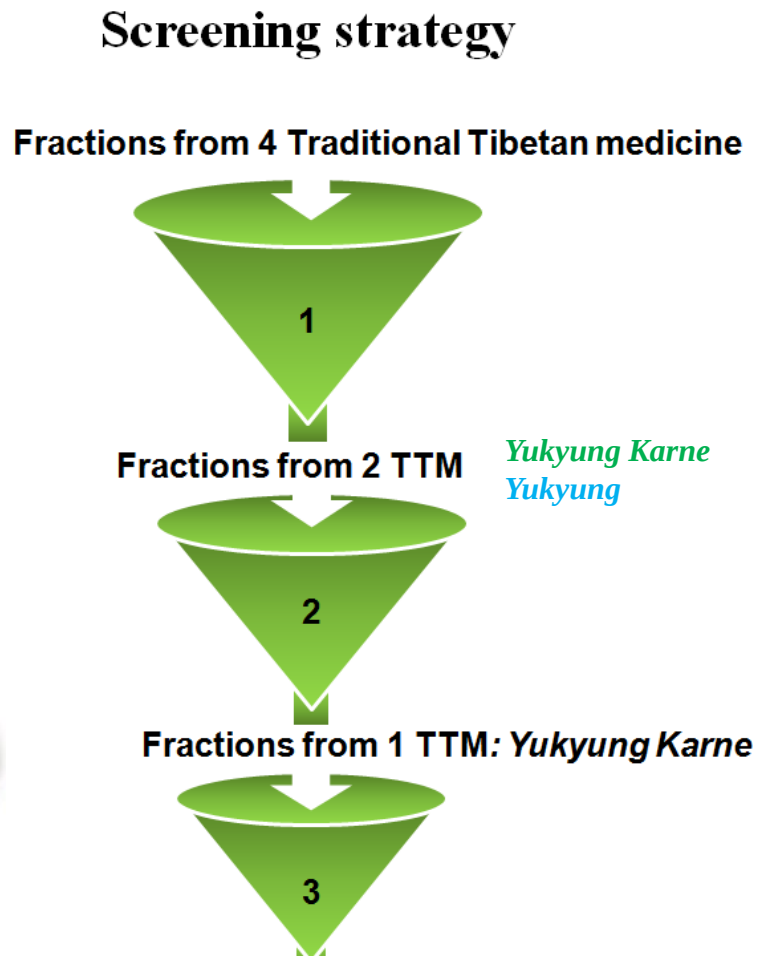
Piper longum

Terminalia chebula

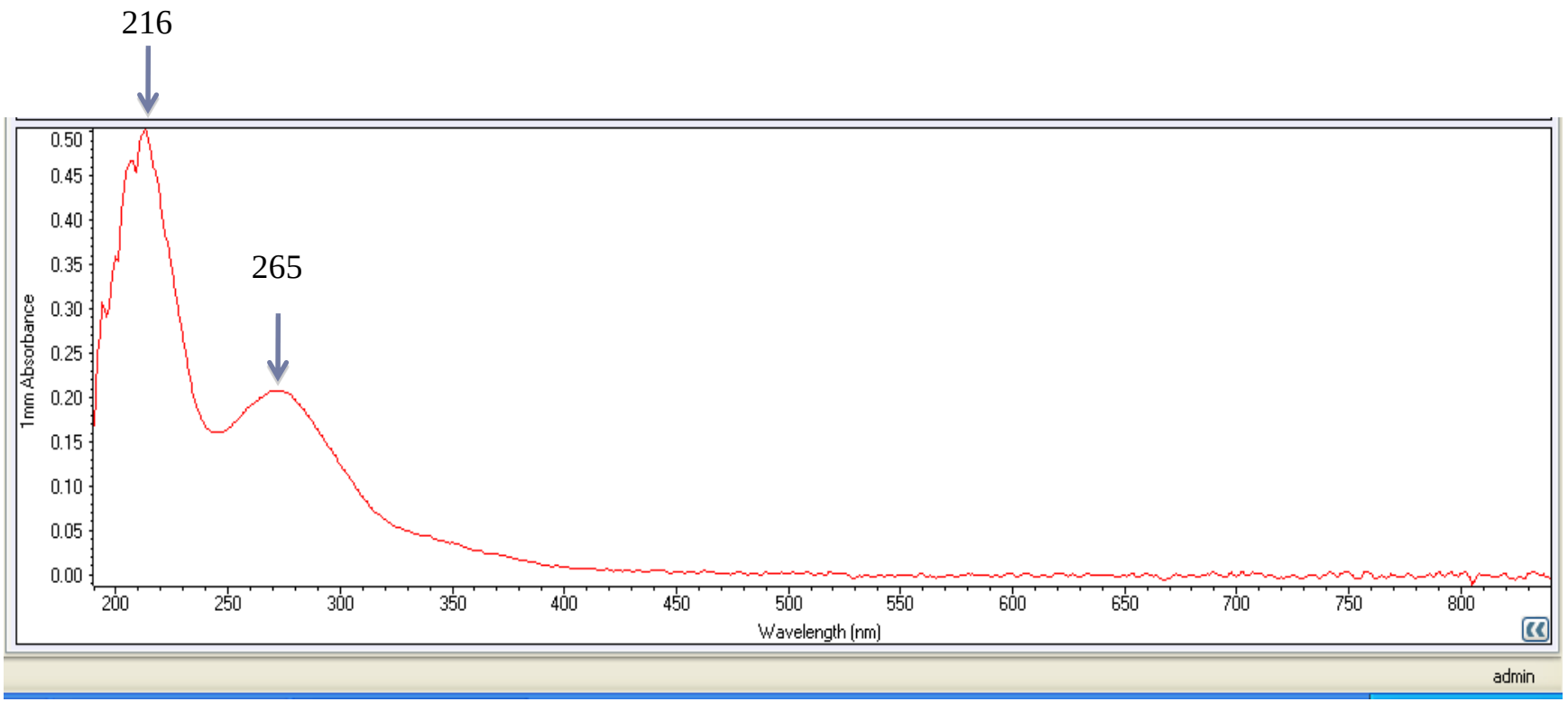
Yukyung Karne (YK)

Anti cancer drug screening

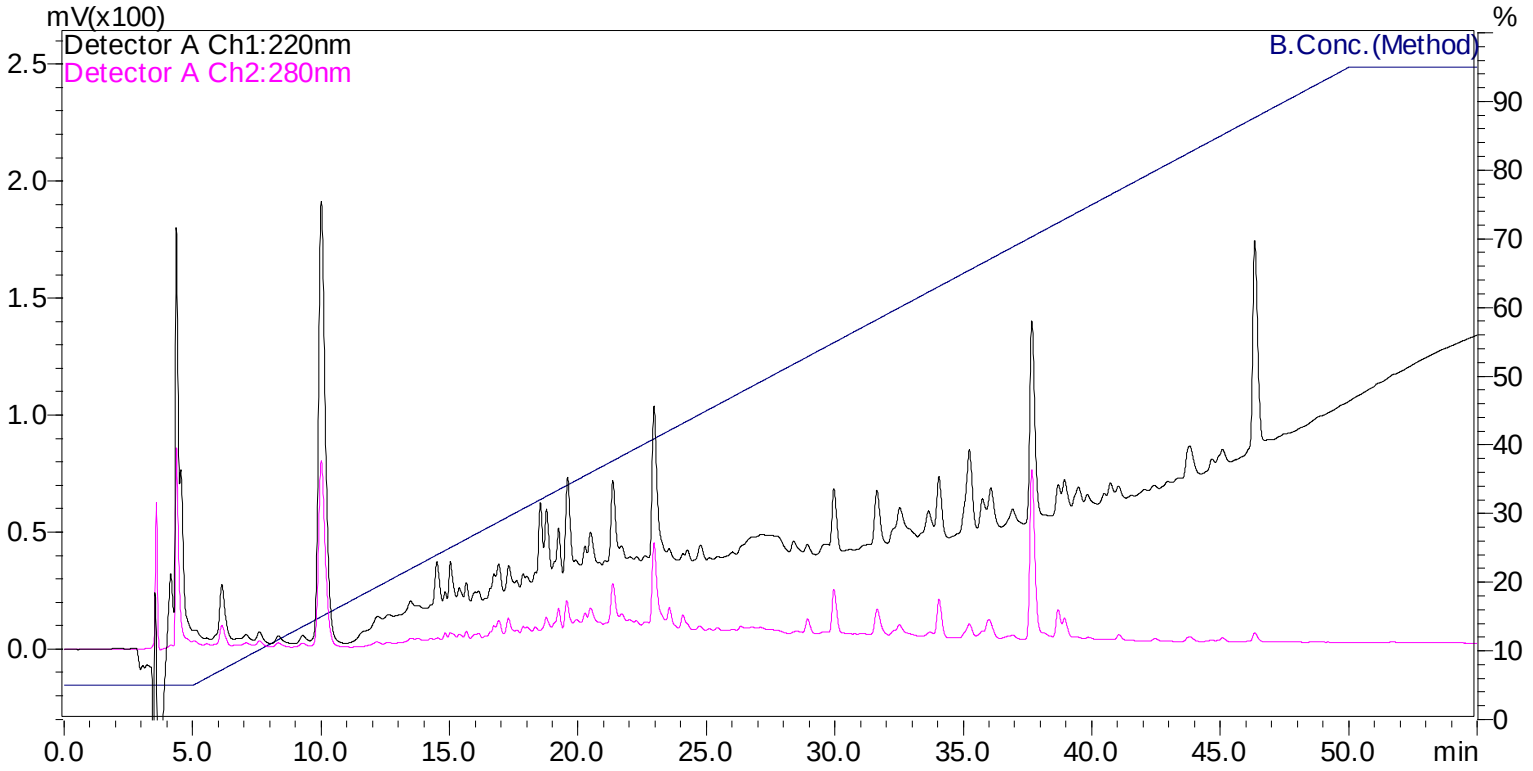




UV- Visible spectroscopy



Analytical RP-HPLC Profile of YK at 220nm and 280nm

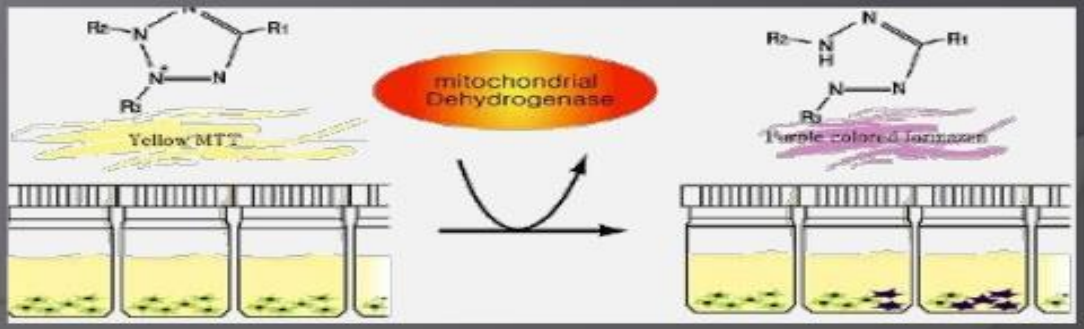


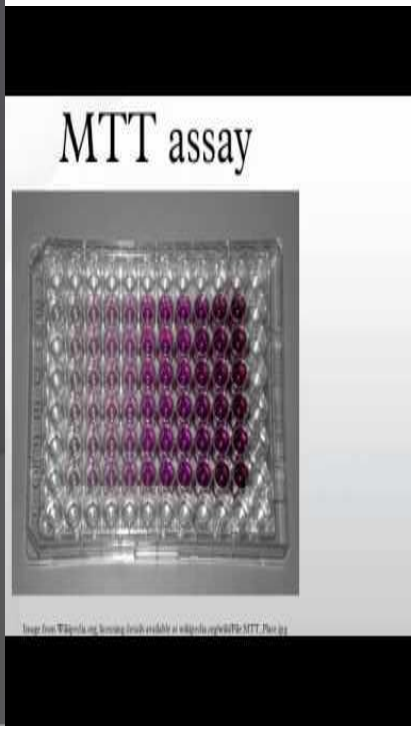
The fingerprints of YK samples were monitored on a Shimadzu reverse-phase HPLC system (C18 column - 250mm, 4.6mm) with SCl-10AVp system controller and SPD-10AVvp UV-Vis detector. The mobile phase (acetonitrile and water) was degassed and filtered through 0.2µm membrane filter before pumping into the HPLC system. A linear gradient of acetonitrile from 5% to 95% over 55 min at a flow rate of 1ml/min was maintained and the samples were monitored at 220 and 280 nm using Photo Diode Array (PDA) detector. The YK samples (20 mg in 200 ml of glass distilled water) were used for injection.

Cell viability-MTT assay

Principle

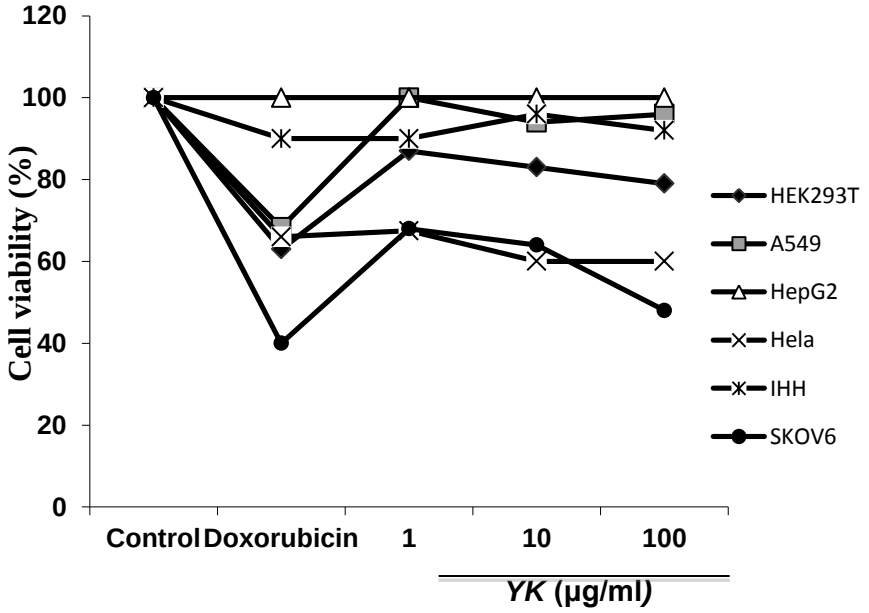
- Water soluble yellow MTT
- Reduced to purple insoluble formazan by mitochondrial dehydrogenases
- Water insoluble formazan can be solubilized using isopropanol or other solvents
- The dissolved material is measured spectrophotometrically yielding absorbance as a function of concentration of converted dye.

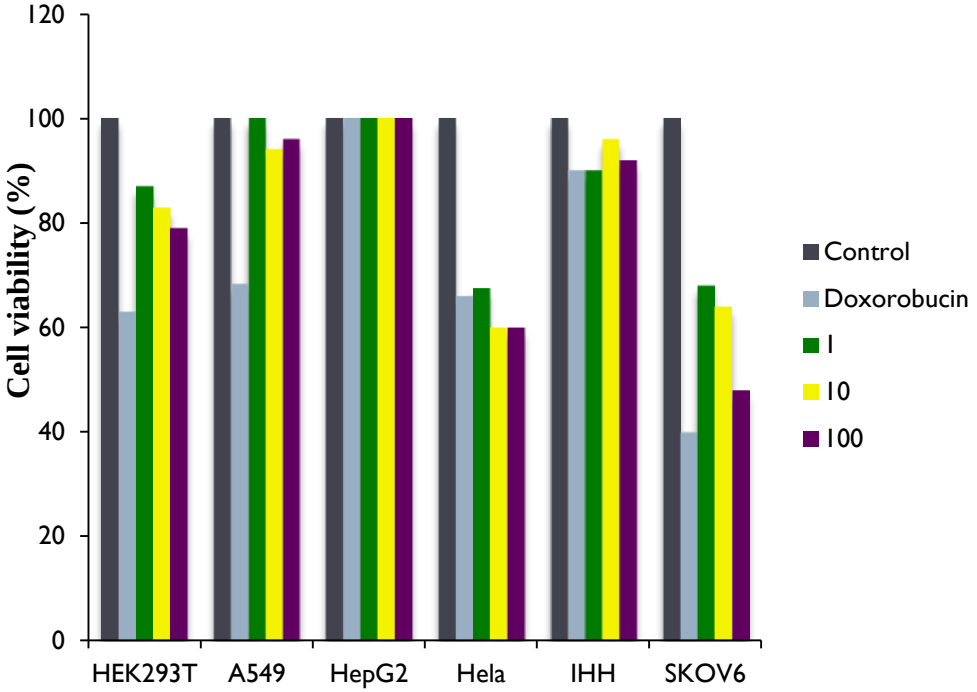




The amount of formazan produces =Directly proportional to number of viable cells in sample.

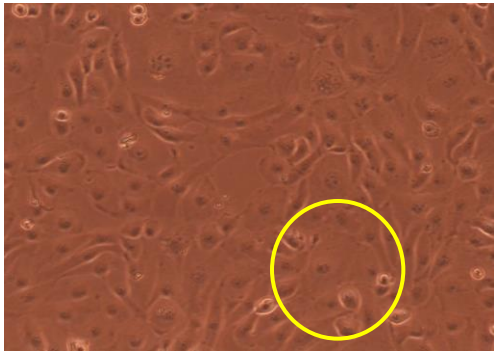
Viability of cells after treatment with *Yukyung Karne* (*YK*)



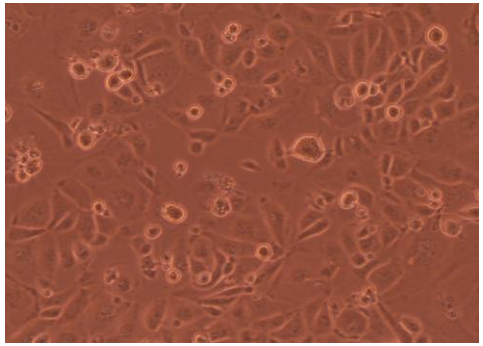


Morphological changes of SKOV6 cell line after *Yukyung Karne* treatment

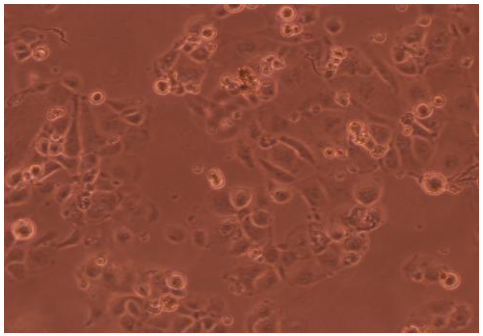
Control



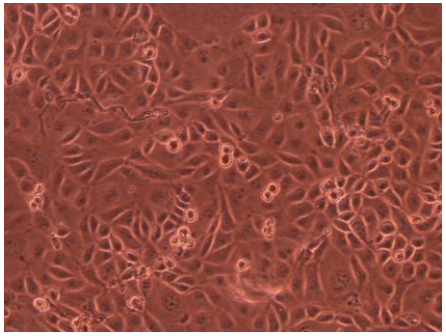
Paclitaxel



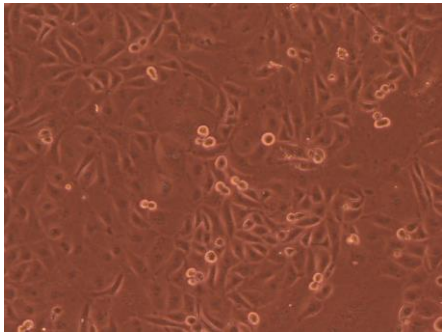
Paclitaxel + YK



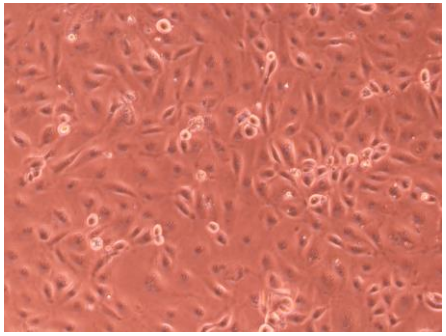
YK 1μg



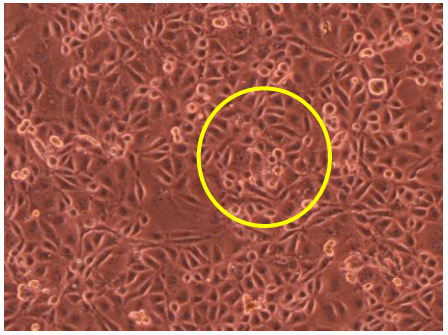
YK10μg



YK 100μg



YK200μg

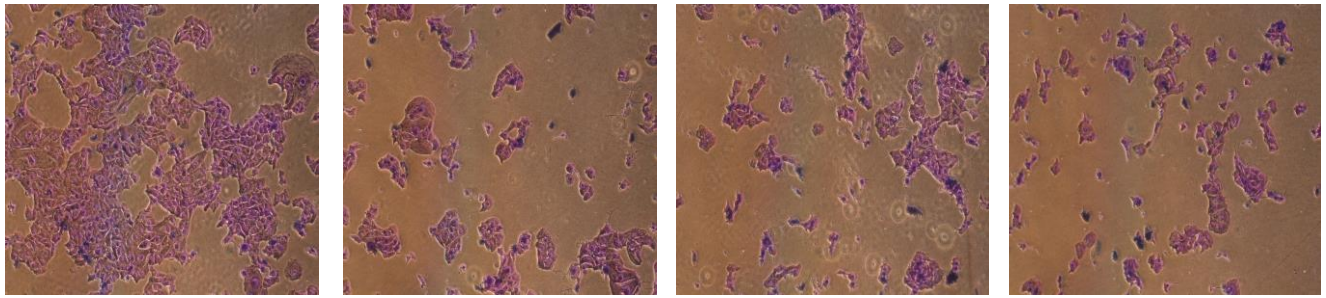


Summary

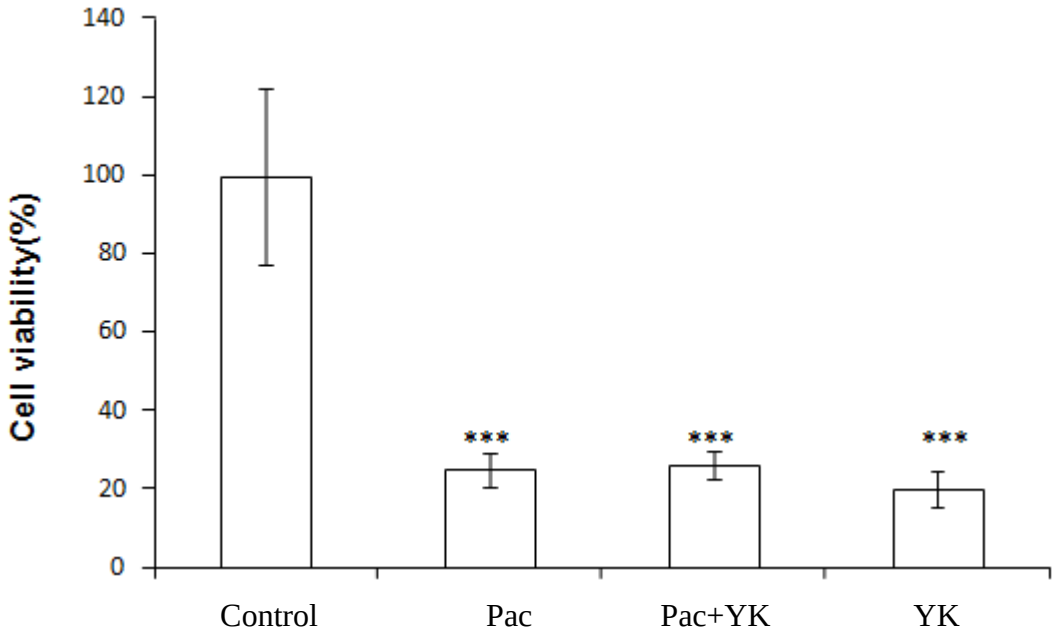
- *Yukyung Karne* was selected after series of screening from four Tibetan Medicine
- Quality of the *Yukyung Karne* composition has been checked by using HPLC and UV while every preparation.
- *Yukyung Karne* induce more cell death in Ovarian cancer compare with other cancer cell lines.
- *Yukyung Karne* is less toxic to normal primary cell line compare to the cancer cell lines.

Yukyung Karne: Induction of apoptosis in ovarian cancer cells

Cell viability assay by crystal violet staining



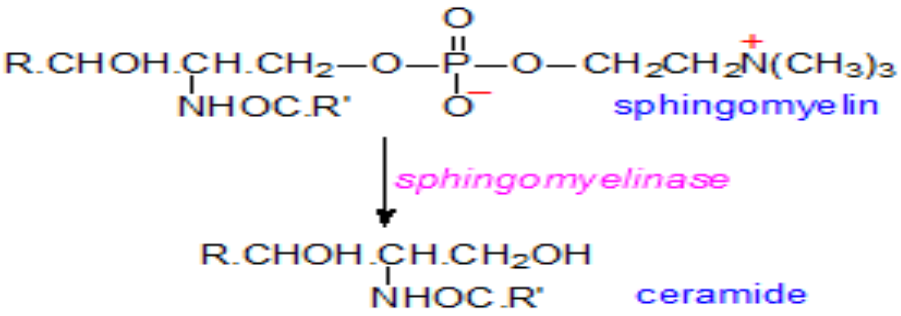
Control Pac Pac+Yk YK

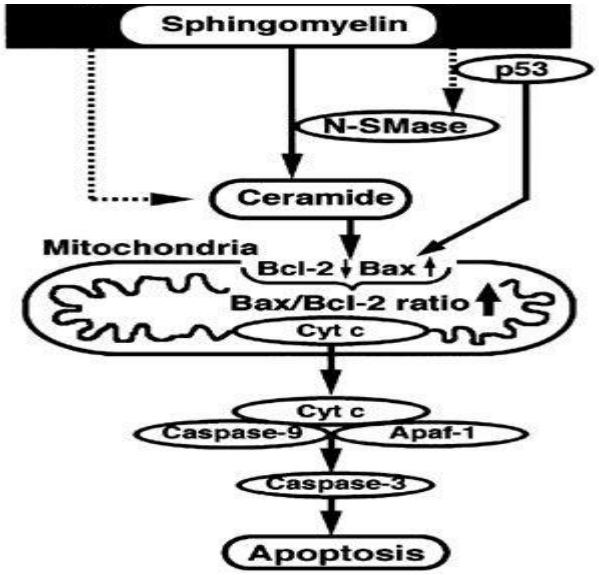


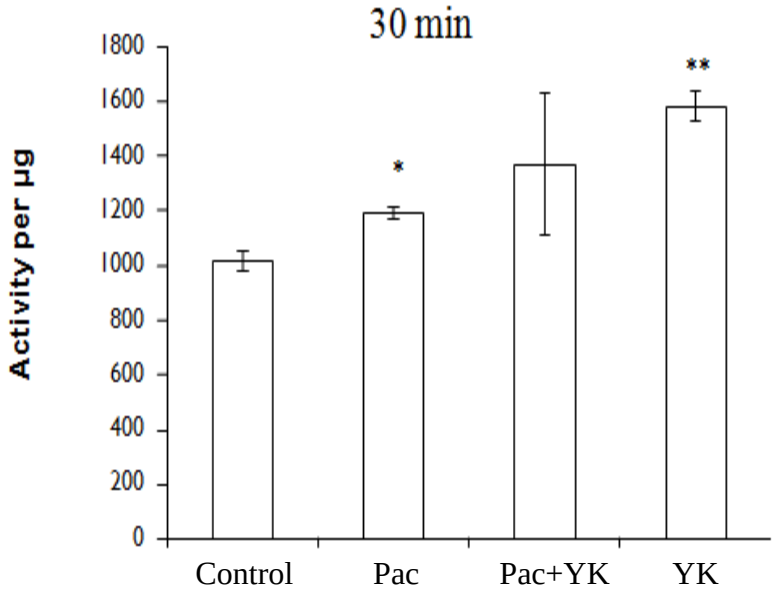
Crystal violet staining stain the nuclei purple

***, P<0.001

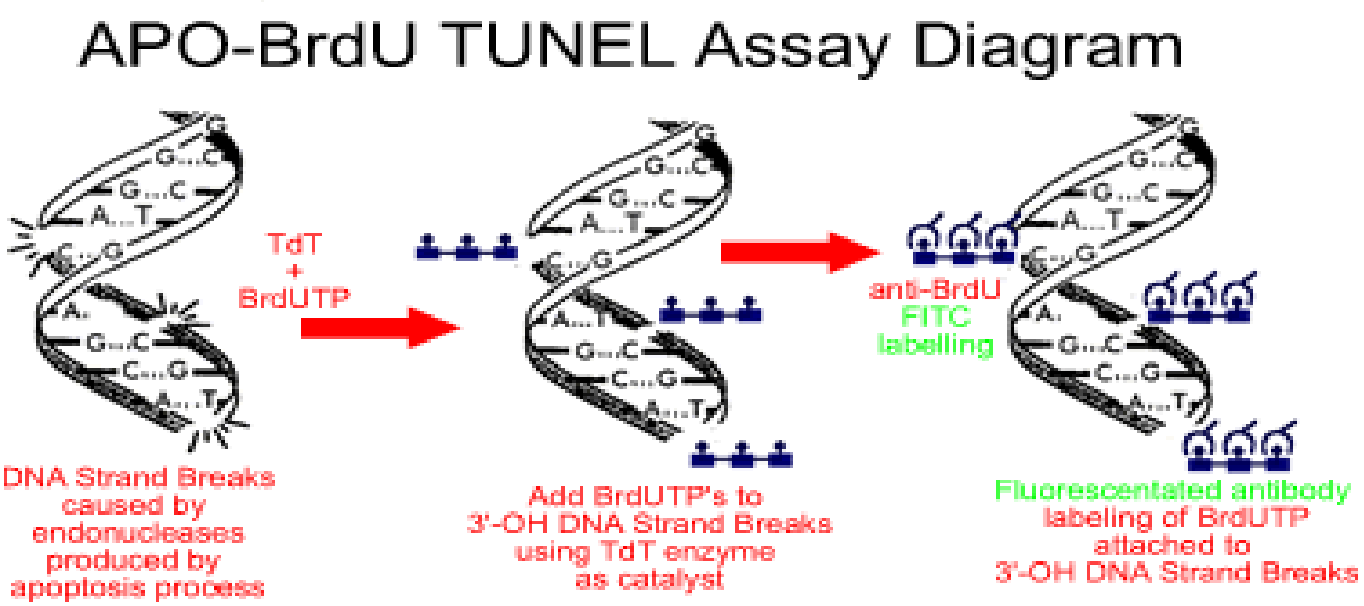
Sphingomyelinase assay





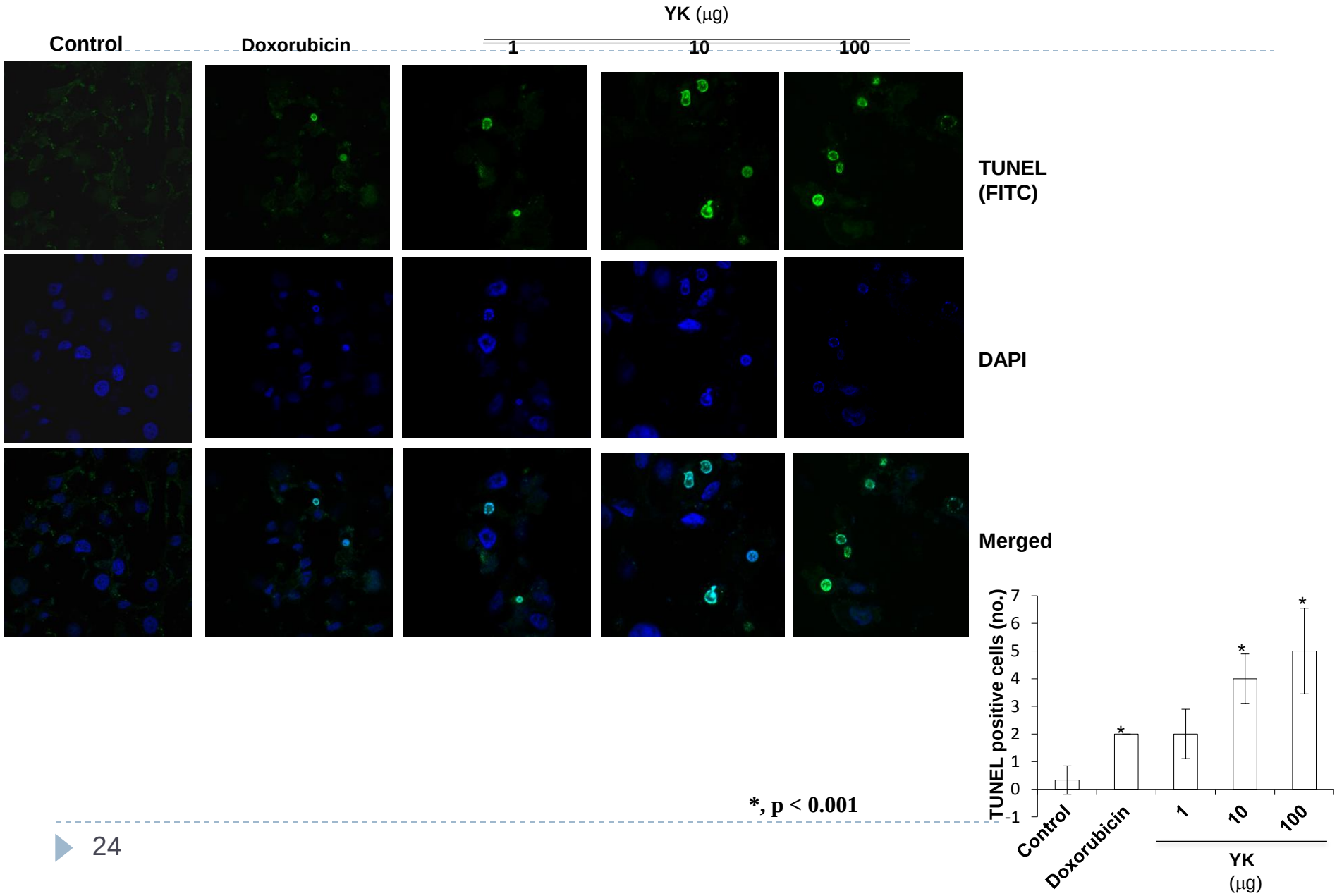


TUNEL ASSAY



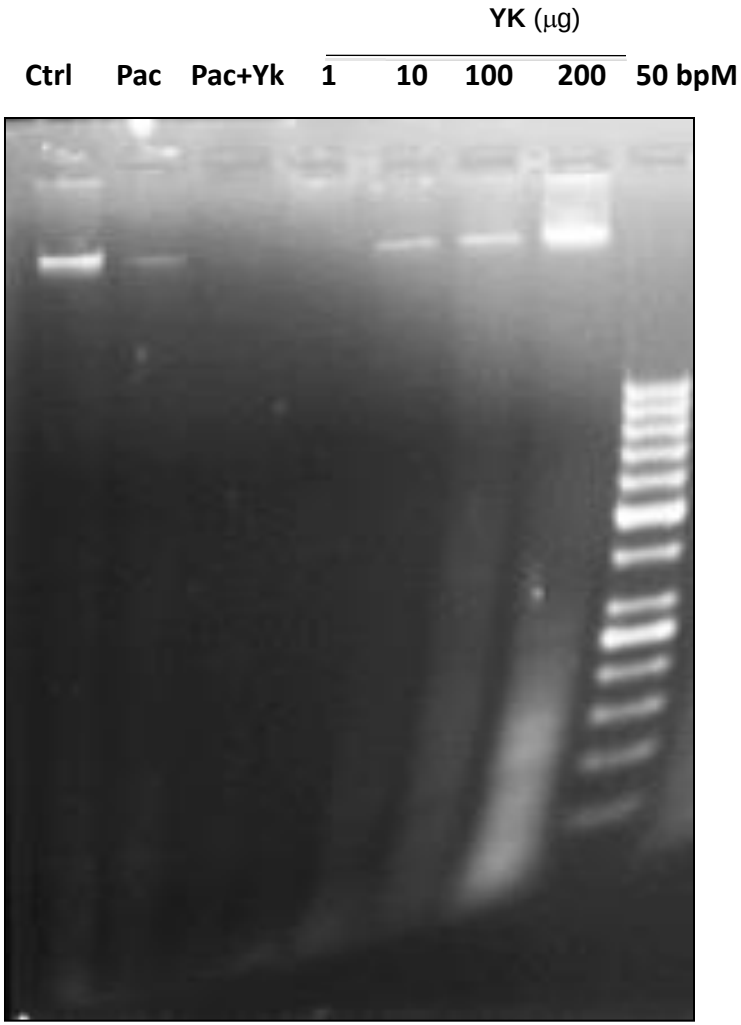
Terminal deoxynucleotidyl transferase (TdT)-mediated addition of a modified dUTP (X-dUTP) to 3'-OH ends of DNA fragments that are generated as a result of apoptosis induction. Incorporated bromoylated dUTP (BrdUTP) is detected by specific antibody conjugates with a reporter enzyme or fluorescent dye

In Situ Cell Death (Apoptosis) Detection-TUNEL assay

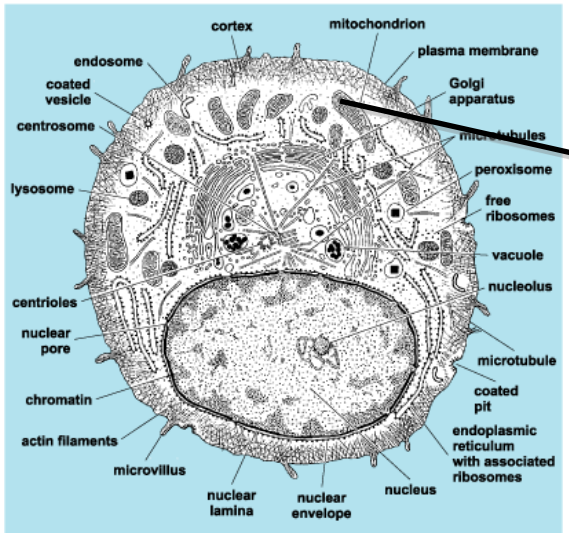


*, $p < 0.001$

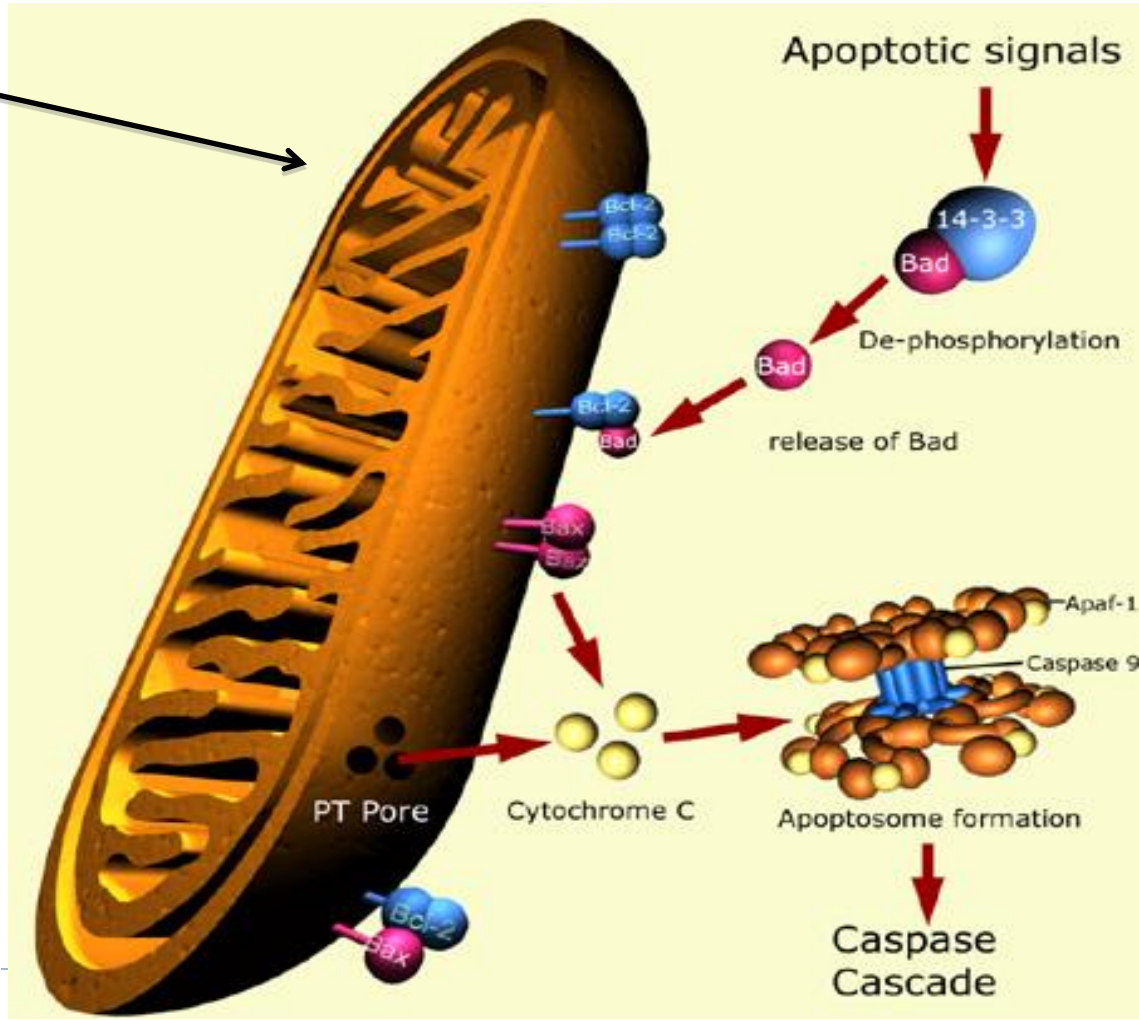
Fragmentation of genomic DNA from SKOV6 cells treated with different concentrations of YK



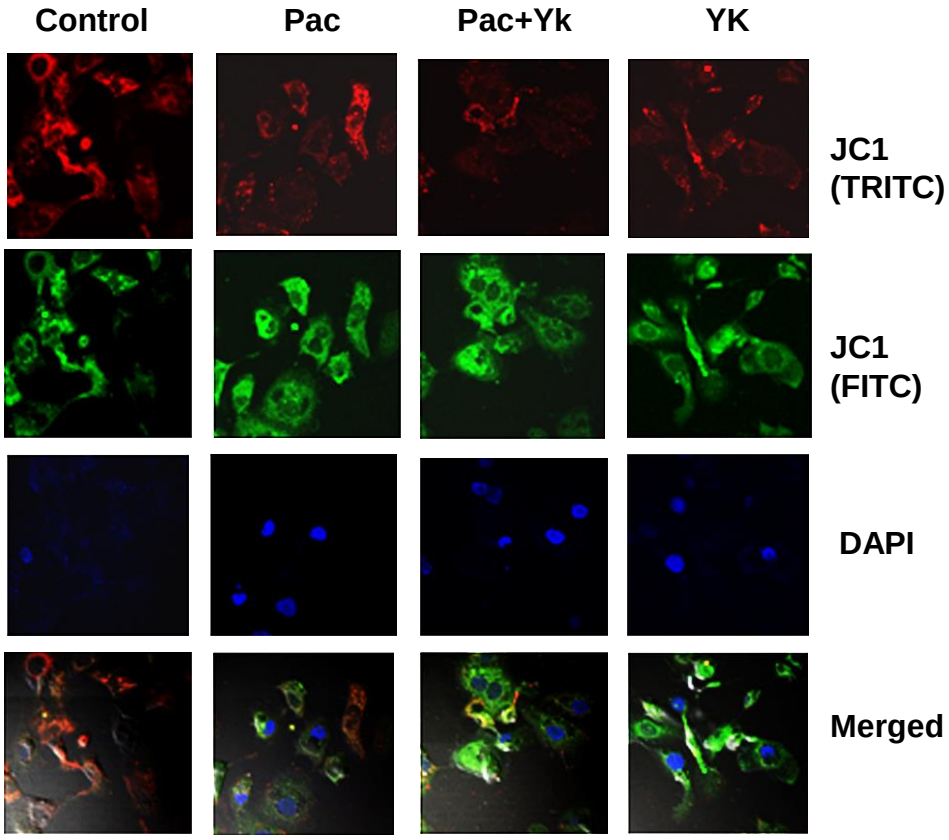
Mitochondria: executor of apoptosis

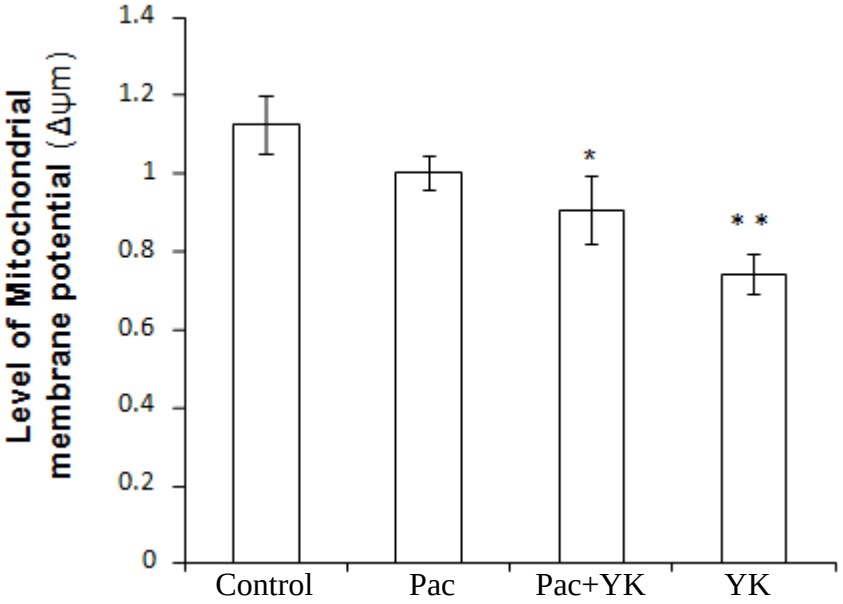


Mitochondria



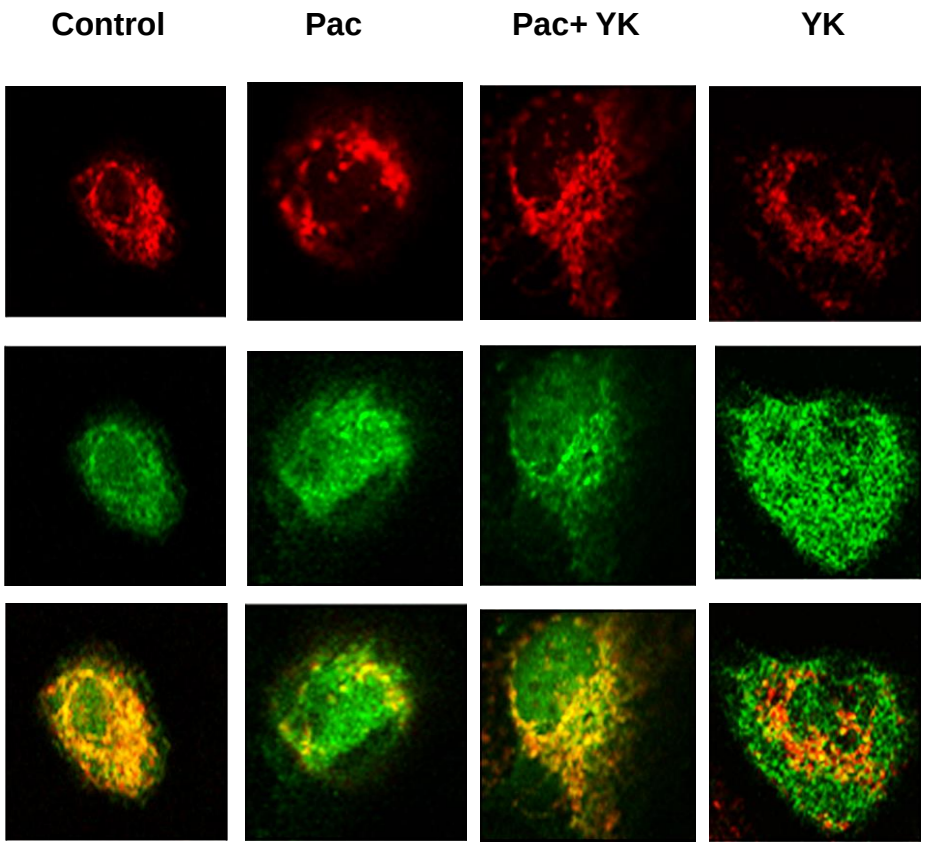
Analysis of mitochondrial membrane potential using JC1 dye

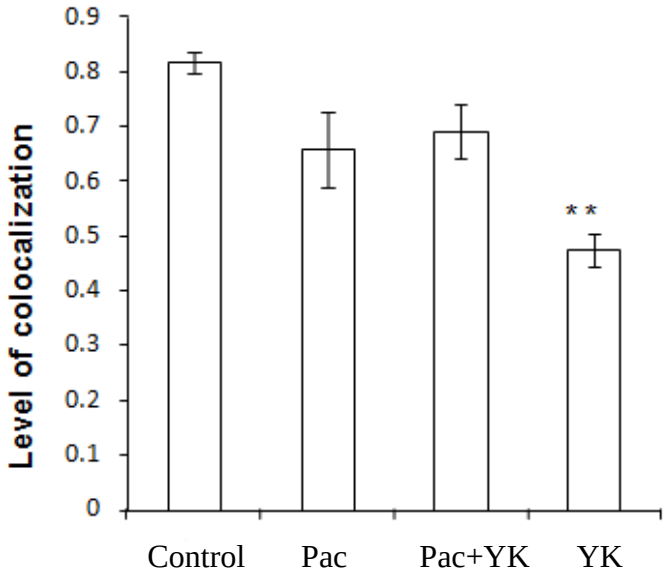




***, p < 0.05; **, p < 0.001.**

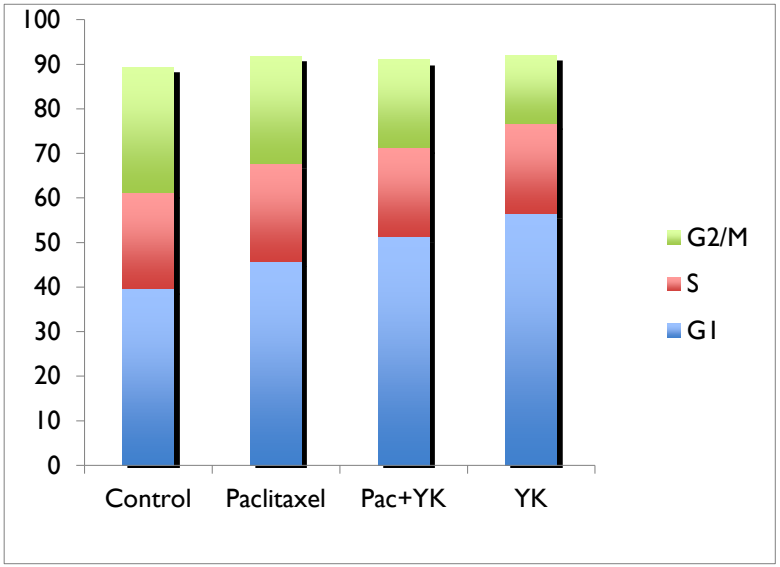
Cellular localisation of Cytochrome C using ds RED mitotracker and GFP Cytochrome C in SKOV 6 cell lines





**, p < 0.001.

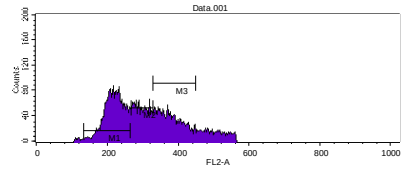
Effect of YK on Cell cycle progression in SKOV6 cells by FACS



Sample	*G1*	*S*	*G2*
Control	59.63 ± 2.0	17.58 ± 2.8	17.83 ± 1.09
Paclitaxel	35.0 ± 9.07*	16.65 ± 2.34	19.93 ± 3.3
Paclitaxel+ YK	38.6 ± 6.38*	17.0 ± 1.5	18.18 ± 3.8
YK	73.4 ± 1.5	15.46 ± 3.09	14.18 ± 0.64

*, Level of significance: p Value< 0.004

Control



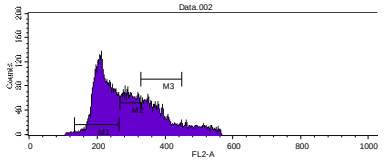
Histogram Statistics

File: Data.001
Sample ID:
Tube: Untitled
Acquisition Date: 01-Jan-70
Gated Events: 13576
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube Lis
Gate: G1
Total Events: 20000

Marker	Left,	Right	Events	% Gated	% Tota	Mean	Geo Mean	CV	Median	Peak Ch
All	0,	102	13576	100.00	67.88	307.51	293.18	31.17	291.00	216
M1	134,	26(	5390	39.70	26.95	219.35	217.67	12.06	220.00	216
M2	268,	32(	2917	21.49	14.59	297.26	296.72	6.06	297.00	319
M3	329,	45(	3820	28.14	19.10	379.05	377.61	8.80	375.00	368

Paclitaxel



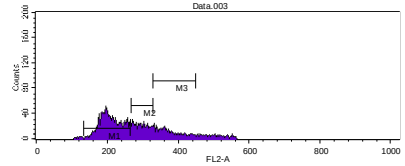
Histogram Statistics

File: Data.002
Sample ID:
Tube: Untitled
Acquisition Date: 01-Jan-70
Gated Events: 16145
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube Lis
Gate: G1
Total Events: 20000

Marker	Left,	Right	Events	% Gated	% Tota	Mean	Geo Mear	CV	Median	Peak Ch
All	0,	102	16145	100.00	80.73	292.27	279.29	30.96	276.00	211
M1	134,	26(	7400	45.83	37.00	216.59	214.98	12.05	214.00	211
M2	268,	32(	3553	22.01	17.77	297.37	296.86	5.90	297.00	282
M3	329,	45(	3874	24.00	19.37	373.56	372.26	8.45	368.00	347

Paclitaxel + YK



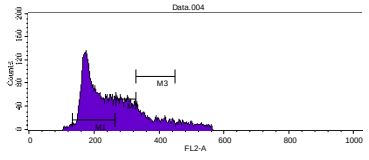
Histogram Statistics

File: Data.003
Sample ID:
Tube: Untitled
Acquisition Date: 01-Jan-70
Gated Events: 5407
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube Lis
Gate: G1
Total Events: 8940

Marker	Left,	Right	Events	% Gated	% Tota	Mean	Geo Mean	CV	Median	Peak Ch
All	0,	102	5407	100.00	60.48	281.53	266.88	33.91	260.00	195
M1	134,	26(	2772	51.27	31.01	209.29	207.32	13.69	205.00	195
M2	268,	32(	1081	19.99	12.09	297.18	296.65	6.02	297.00	270
M3	329,	45(	1065	19.70	11.91	375.24	373.76	9.03	367.00	332

YK



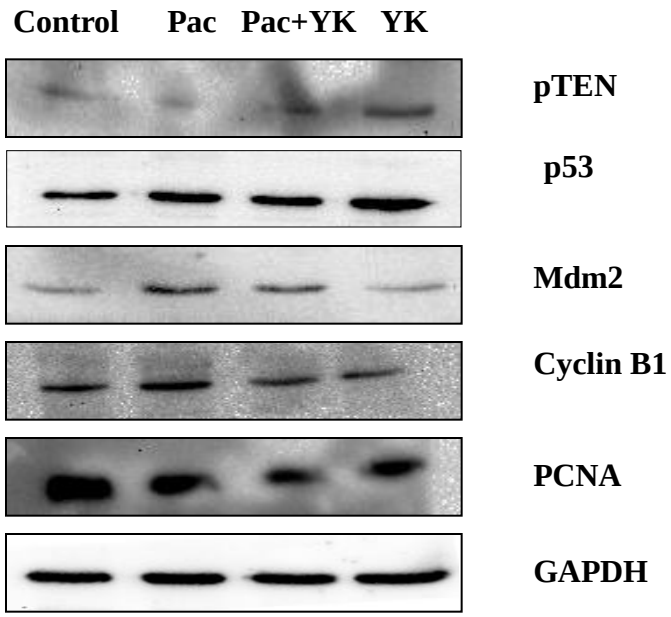
Histogram Statistics

File: Data.004
Sample ID:
Tube: Untitled
Acquisition Date: 01-Jan-70
Gated Events: 13881
X Parameter: FL2-A (Linear)

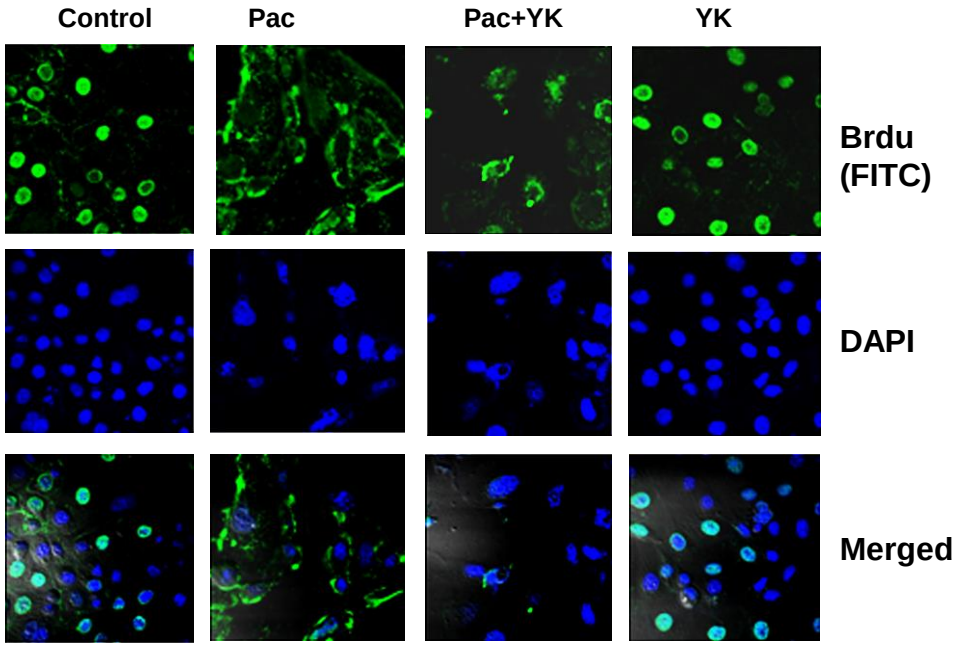
Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube Lis
Gate: G1
Total Events: 20000

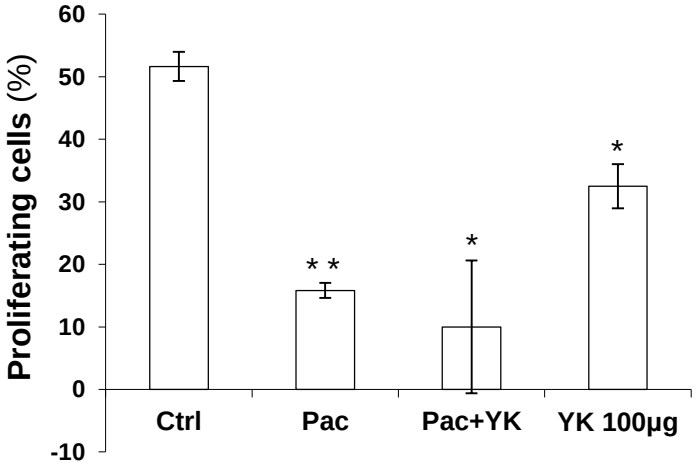
Marker	Left,	Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0,	102	13881	100.00	69.41	266.70	251.17	36.25	245.00	178
M1	134,	26(	7852	56.57	39.26	199.99	197.46	16.09	193.00	178
M2	268,	32(	2789	20.09	13.94	297.63	297.11	5.94	297.00	281
M3	329,	45(	2136	15.39	10.68	378.31	376.61	9.56	372.00	331

Effects of *Yukyung Karne* on tumor suppressor and cell cycle regulator



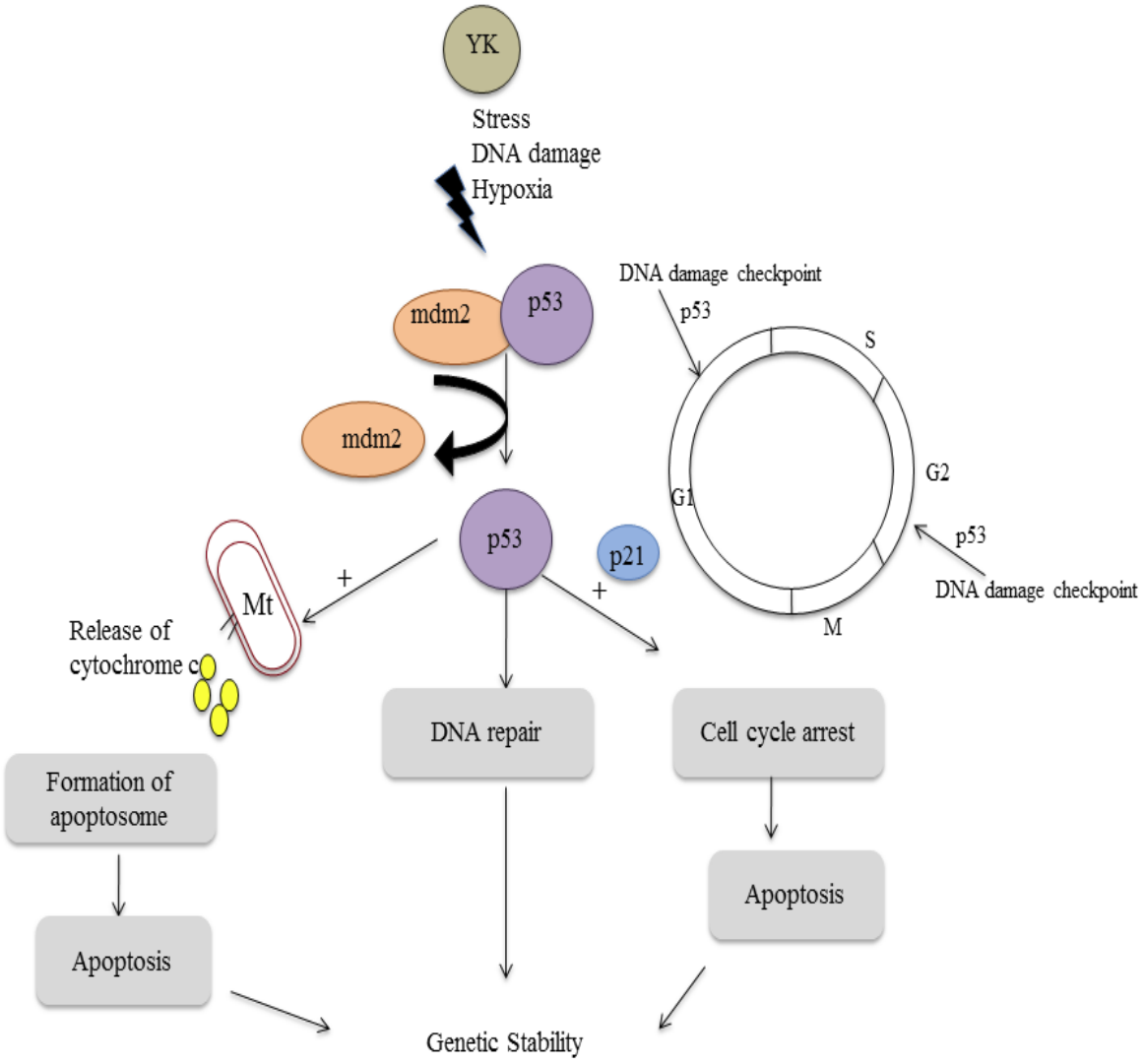
Brdu incorporation-cell proliferation assay





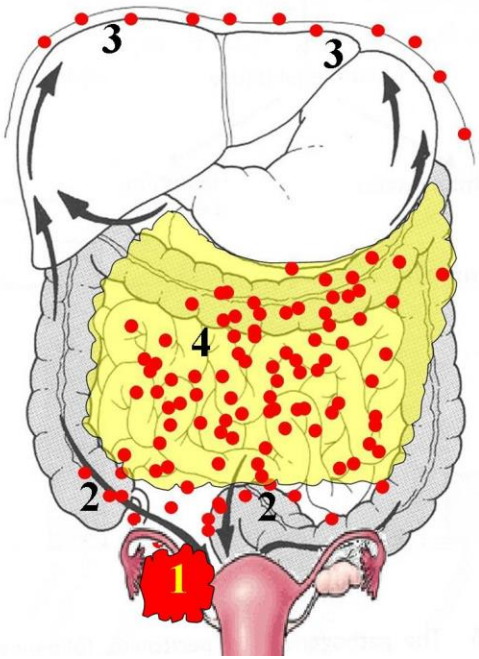
*, p < 0.02, **, p < 0.002.

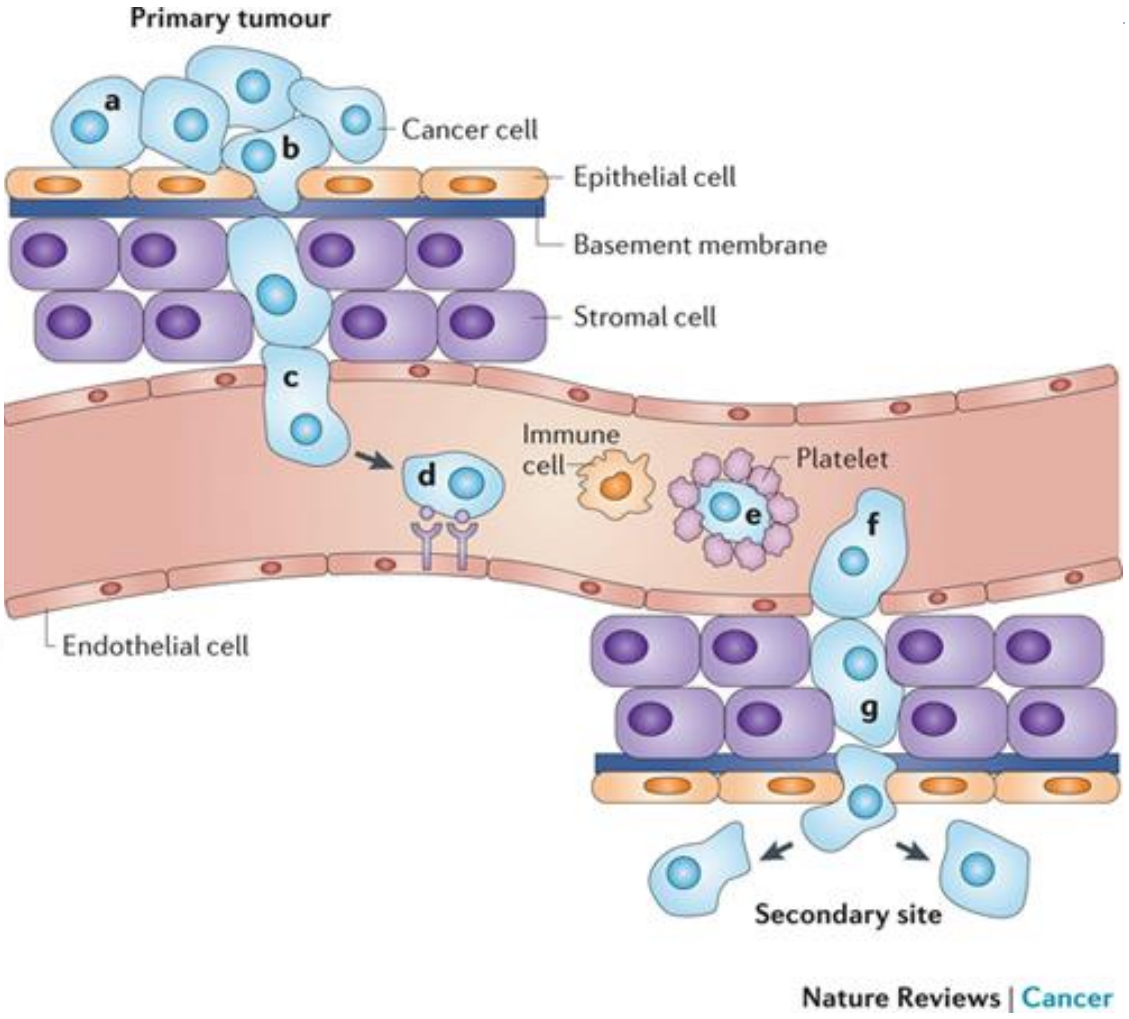
- *Yukyung Karne* had cytotoxic as well as antiproliferative properties in ovarian cancer cells
- *Yukyung Karne* induce cell cycle arrest at G1 phase
- *Yukyung Karne* induces efflux of Cytochrome C and activates mitochondrial dependent apoptosis in ovarian cancer cells
- *Yukyung Karne* restore tumor suppressor p53 and pTEN expression
- *Yukyung Karne* and paclitaxel have synergistic effects on induction of apoptosis



Effect of *Yukyung Karne* on migration and transformation of ovarian cancer cells

Metastasis-Overview

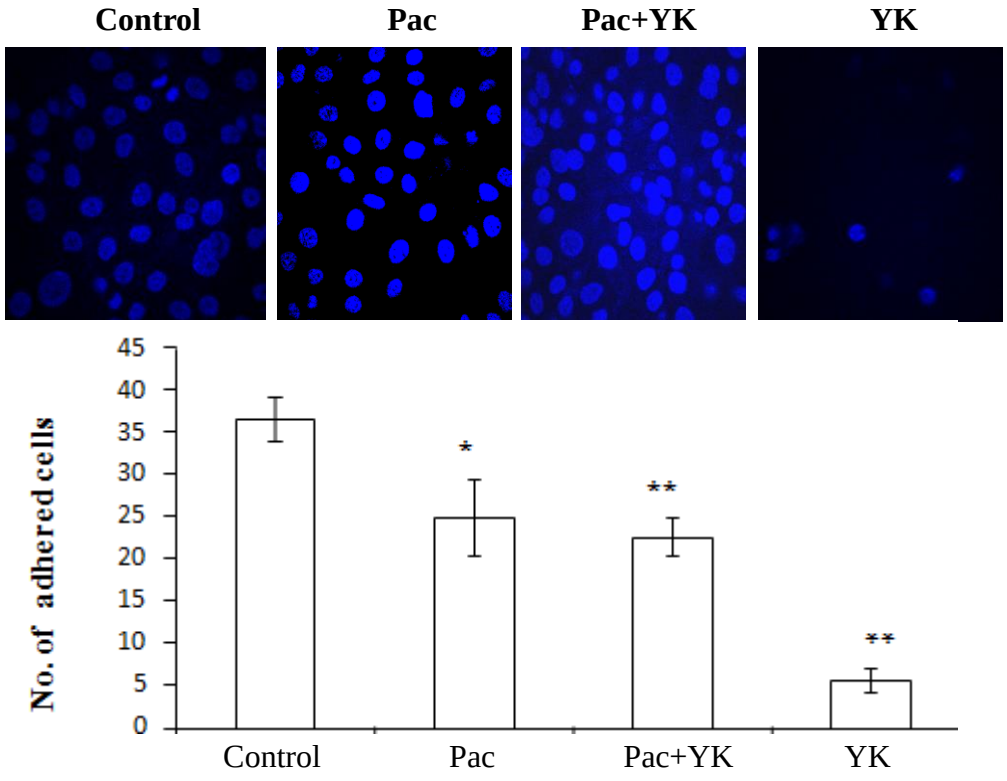




Steps
*Shedding of cells from primary tumor
*Entrance into the vascular system
*Travel to distant site
*Growth
*Angiogenesis

Effect of *Yukyung Karne* on tumor cell adhesion to ECM protein Collagen

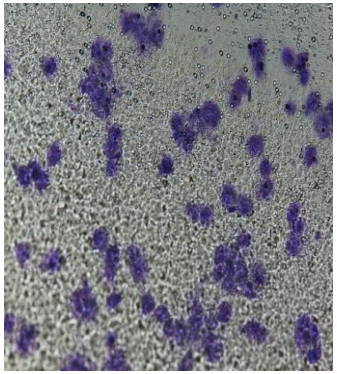
- Most abundant protein
- Significant role in cancer progession
- Determines the spread of metastatic cells



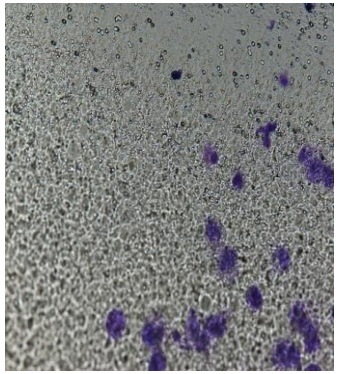
***, P<0.05, **, P<0.001**

Effect of *Yukyung Karne* on tumor cell invasion and angiogenesis

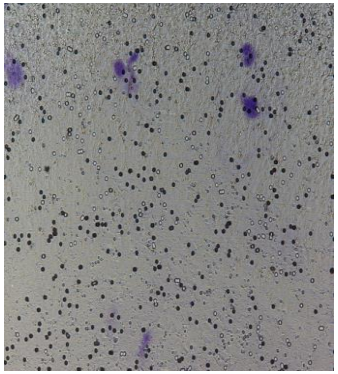
Control



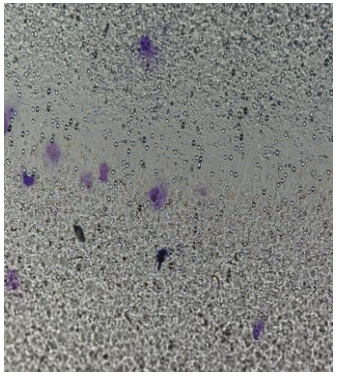
Paclitaxel



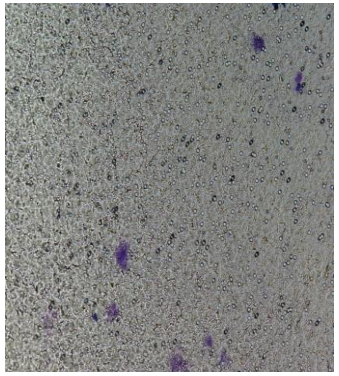
Pac+YK



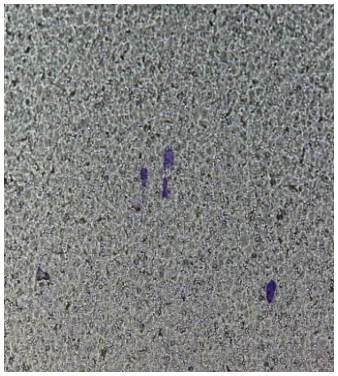
YK50μg



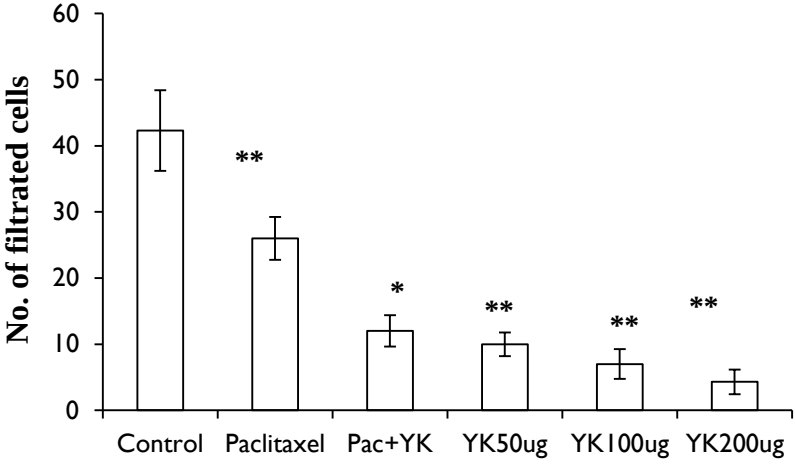
YK100μg



YK200μg



Cell invasion studies



:*, P<0.05, **, P<0.001

Sample	VEGF ± SEM (pg/ml)
Control	2.6±0.057
Paclitaxel	2.10±0.01*
Paclitaxel+YK	1.84±0.02*
YK	1.53±0.05*

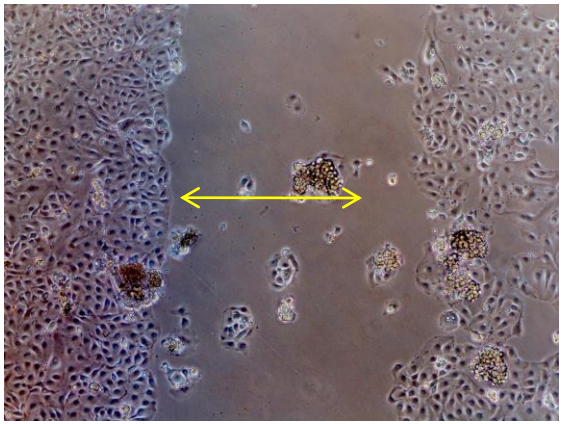
*, Level of significance p Value < 0.001

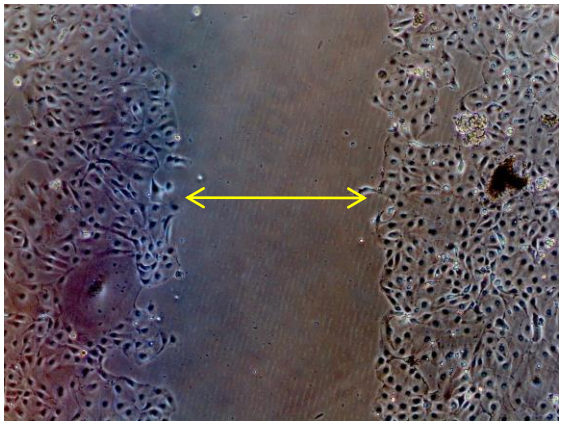
Effect of YK on VEGF secretion from SKOV6 cells

Effect of *Yukyung Karne* on cancer cell migration

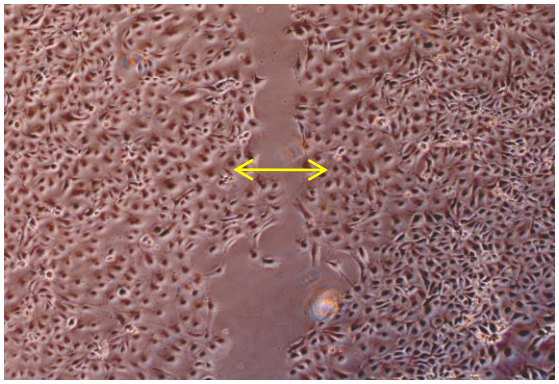
Control

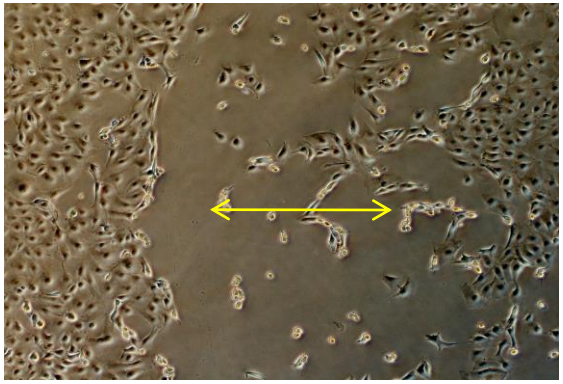
YK





6h

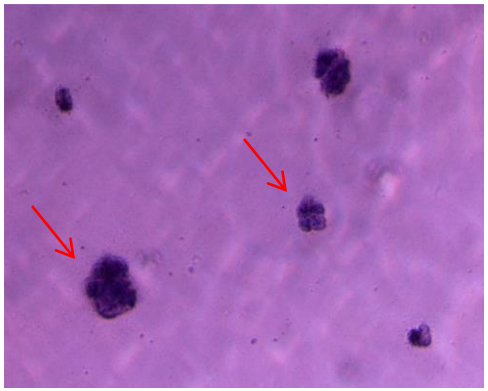




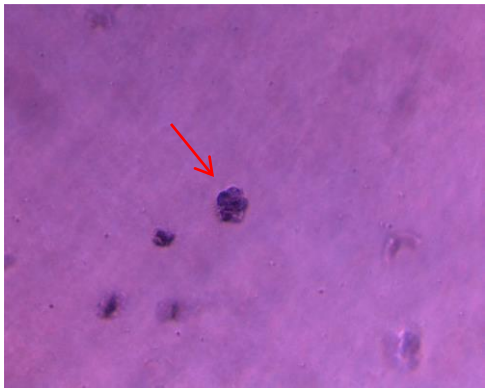
24h

Effect of *Yukyung Karne* on Colony formation

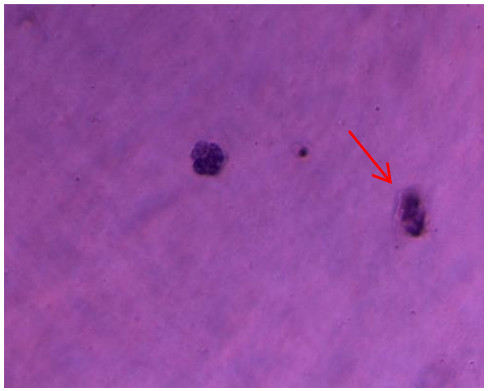
Control



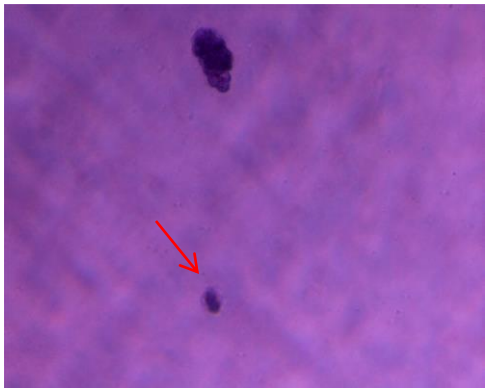
Pac

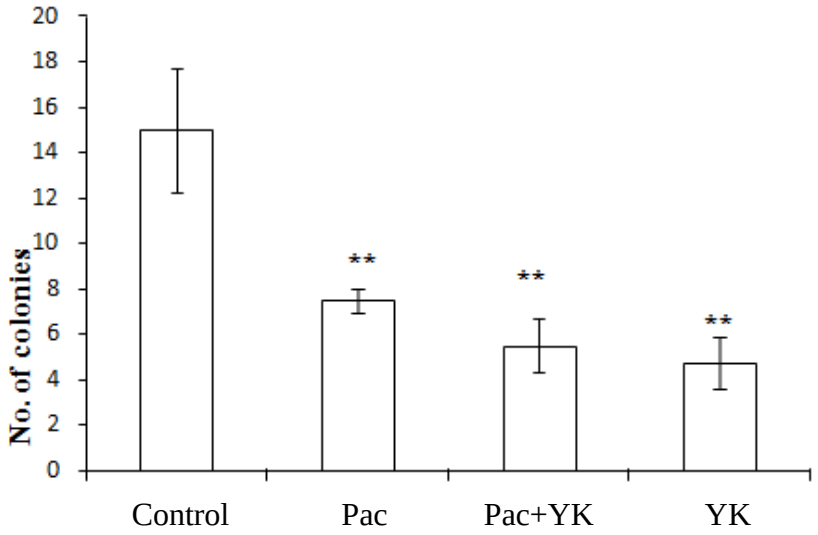


Pac+YK



YK





**, P<0.001

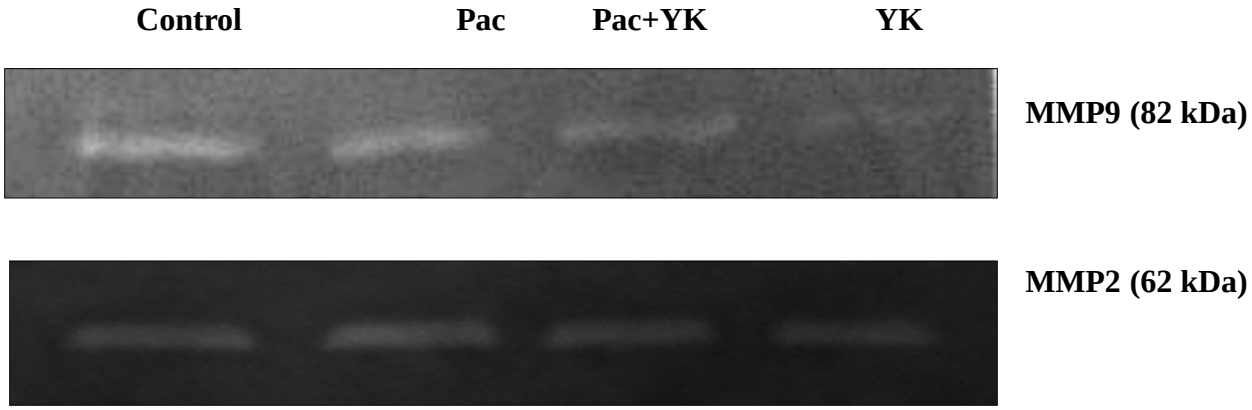
Summary

- *Yukyung Karne* inhibited adhesion, invasion and migration of ovarian cancer cells.
- *Yukyung Karne* suppresses colony formation/transformation of ovarian cancer cells.
- *Yukyung Karne* showed anti angiogenesis property

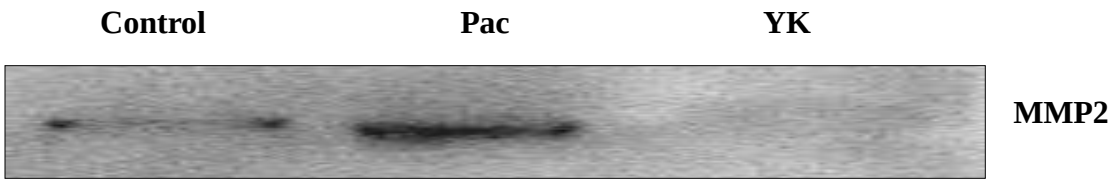
Effect of *Yukyung Karne* on Extracellular matrices and Epithelial to mesenchymal transition

Effect of *Yukyung Karne* on Matrix metalloproteases

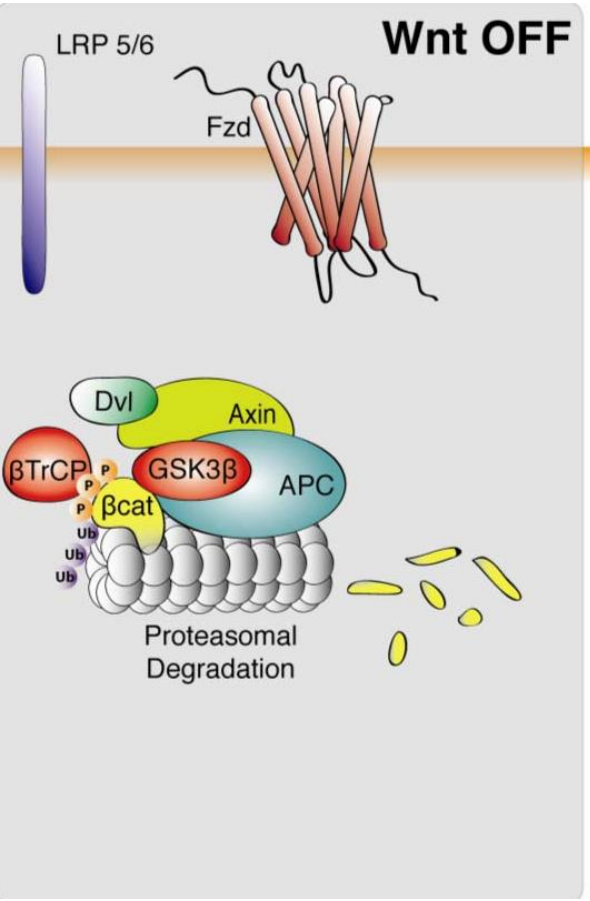
Gelatin Zymography

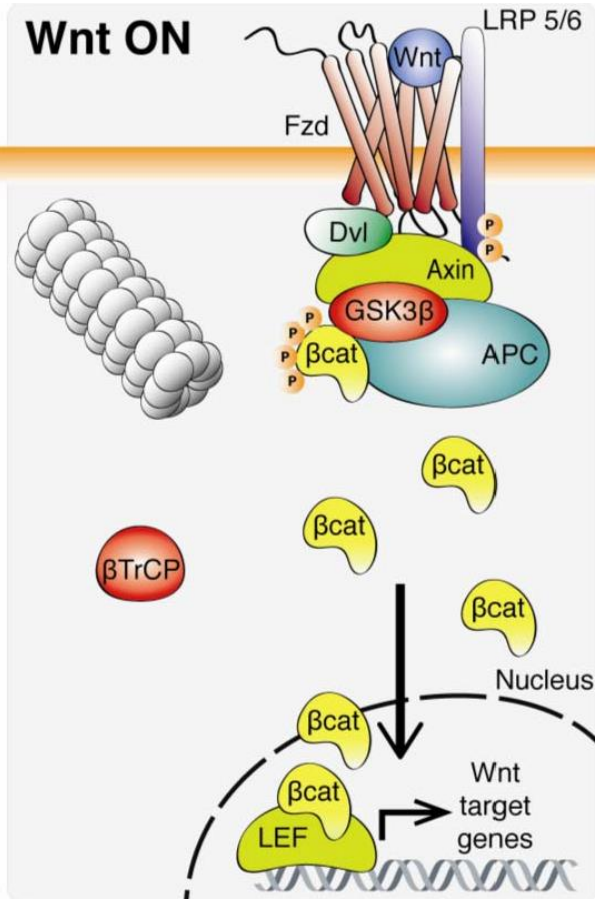


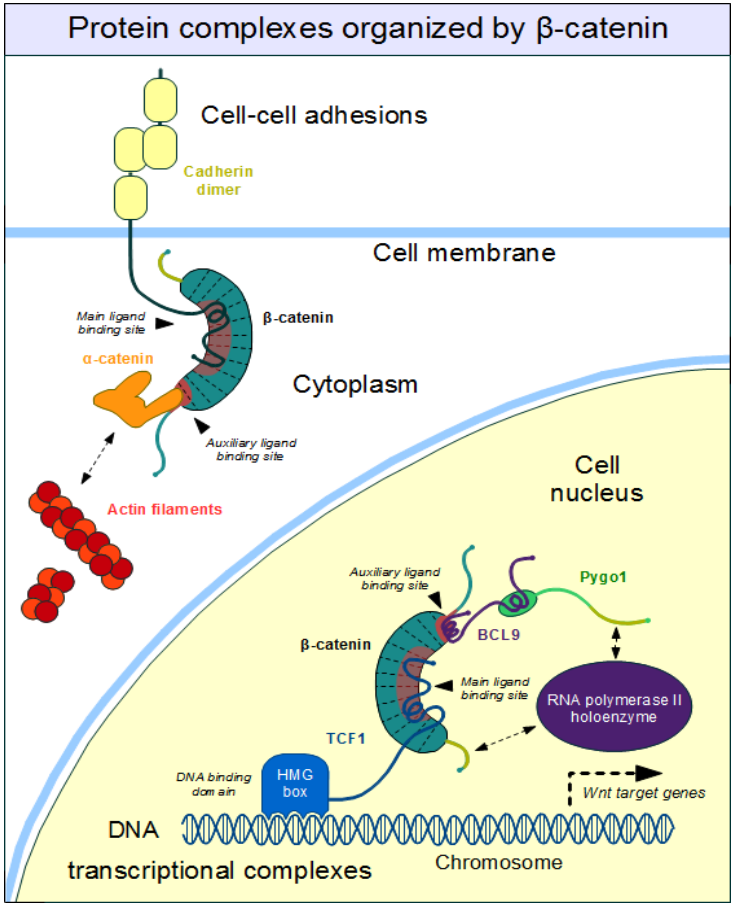
Western blotting



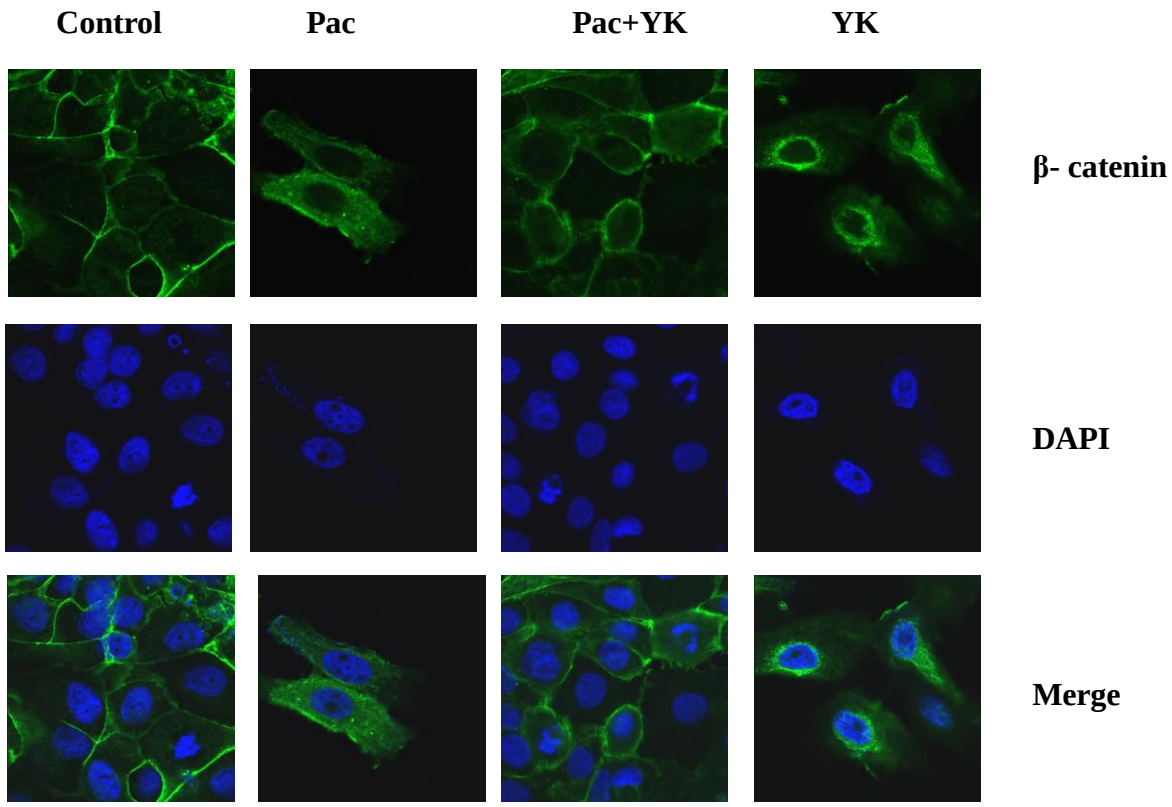
Wnt -ß catenin pathway





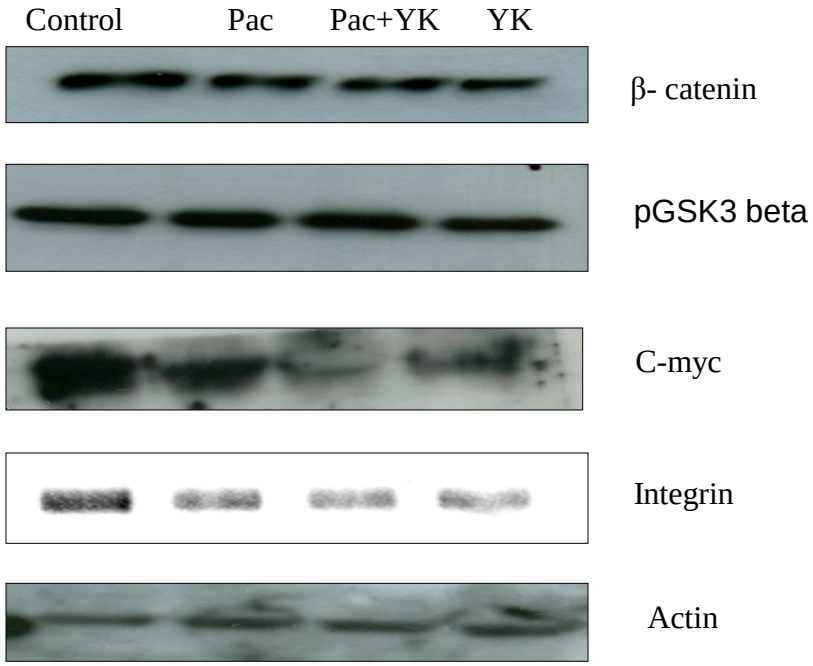


Level of ECM components- β-catenin (92kda)



ECM components: Integrin

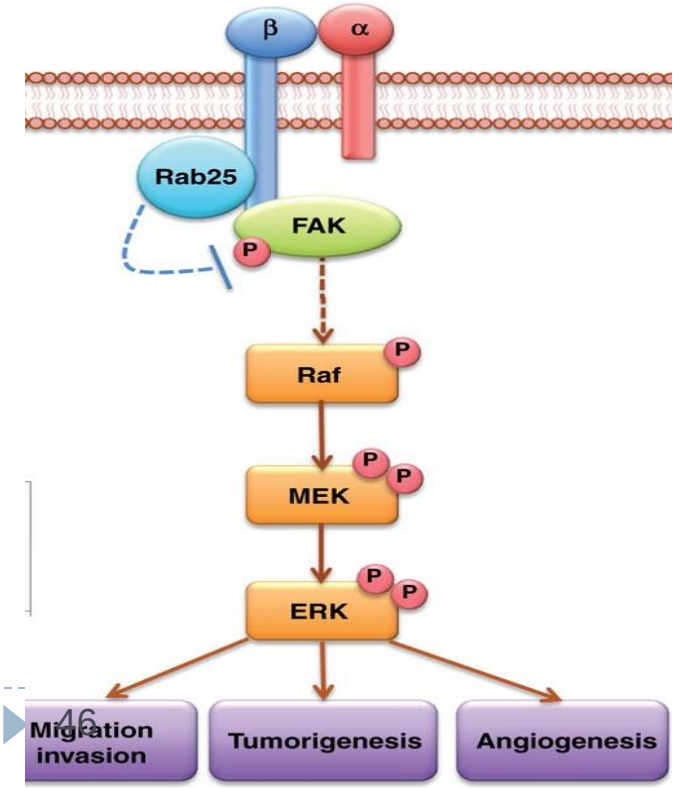
- Transmembrane cell surface receptor
- Regulation of proliferation, invasiveness and chemotherapy resistance
- Integrin binds to ECM and contributes to invasion and migration
- Low survival in clinically overexpressed Integrin patient

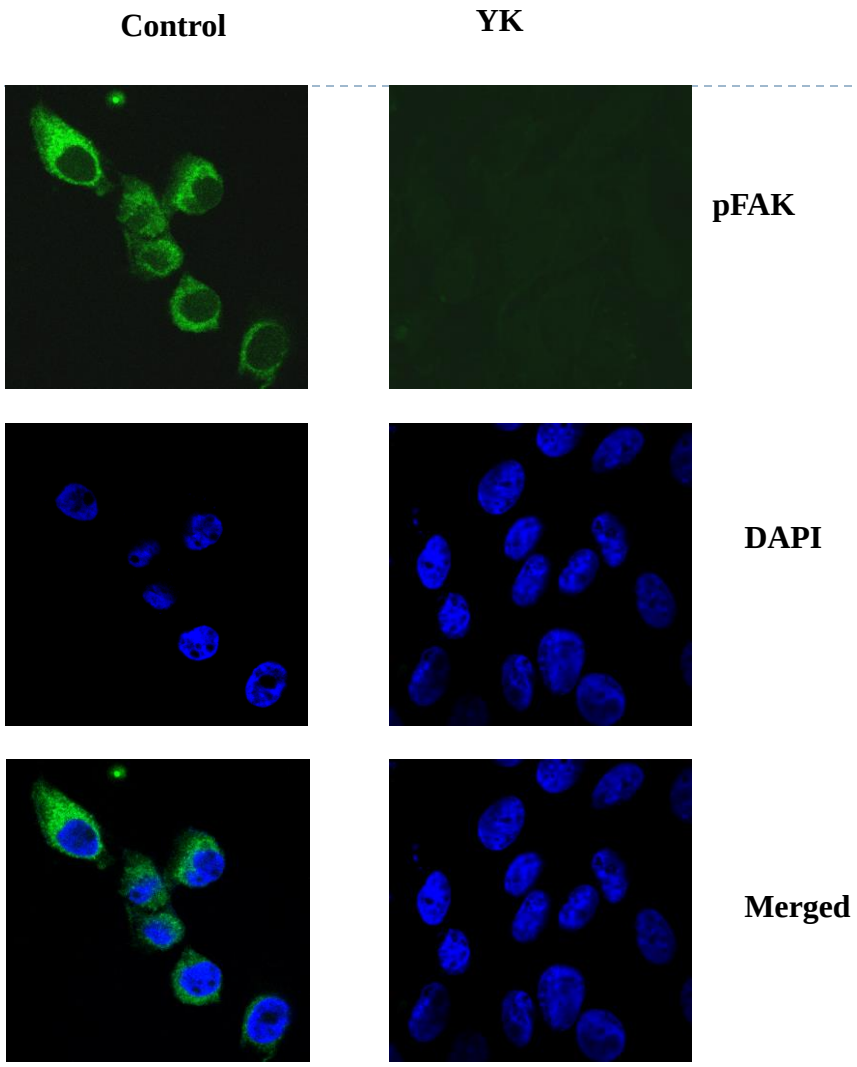


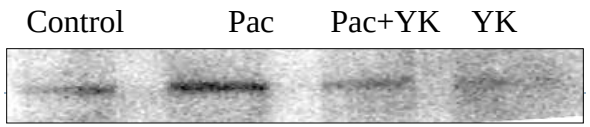
Level of ECM components- pFAK

ECM components: pFAK

- 125Kda non receptor kinase
- Binds to cytoplasmic tail of integrin
- Transmits signal from Integrin and growth factors
- Overexpressed and hyperphosphorylated in cancer

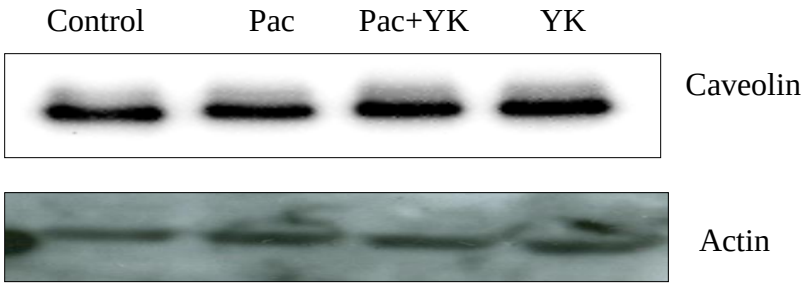


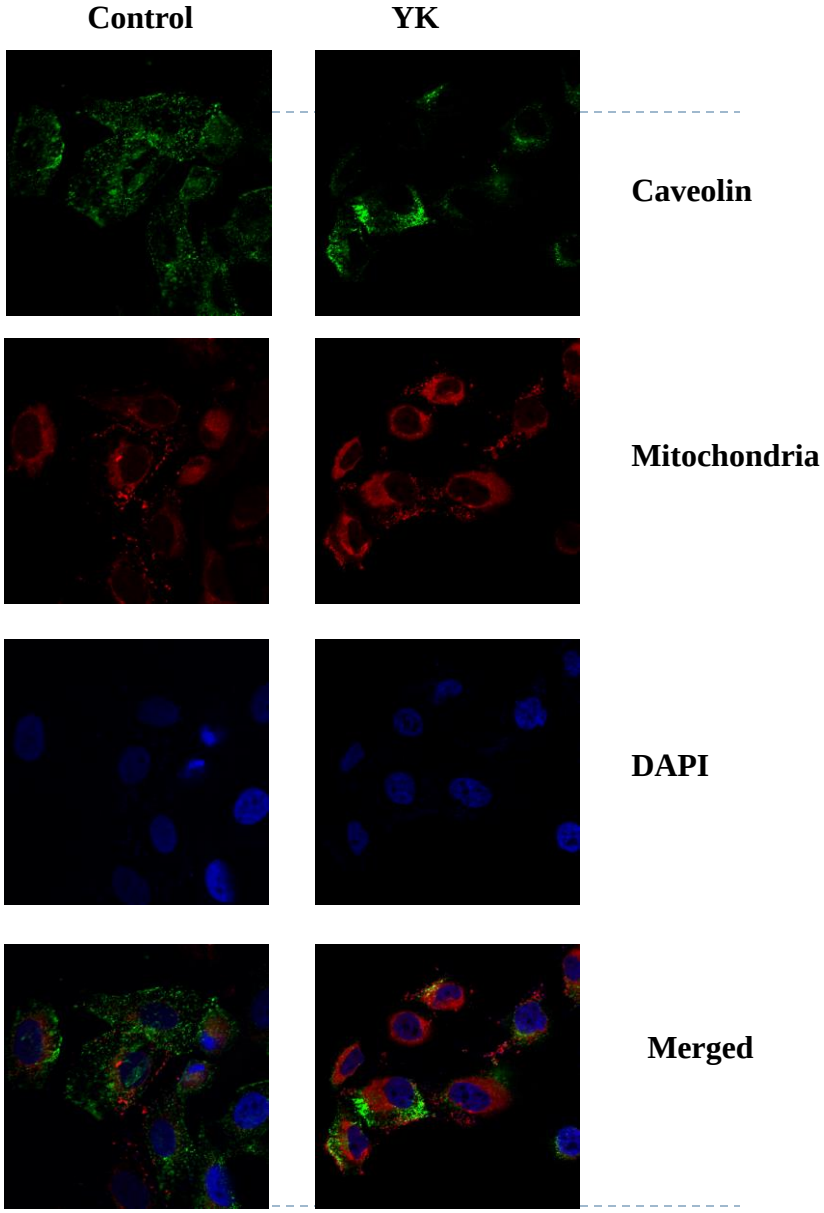




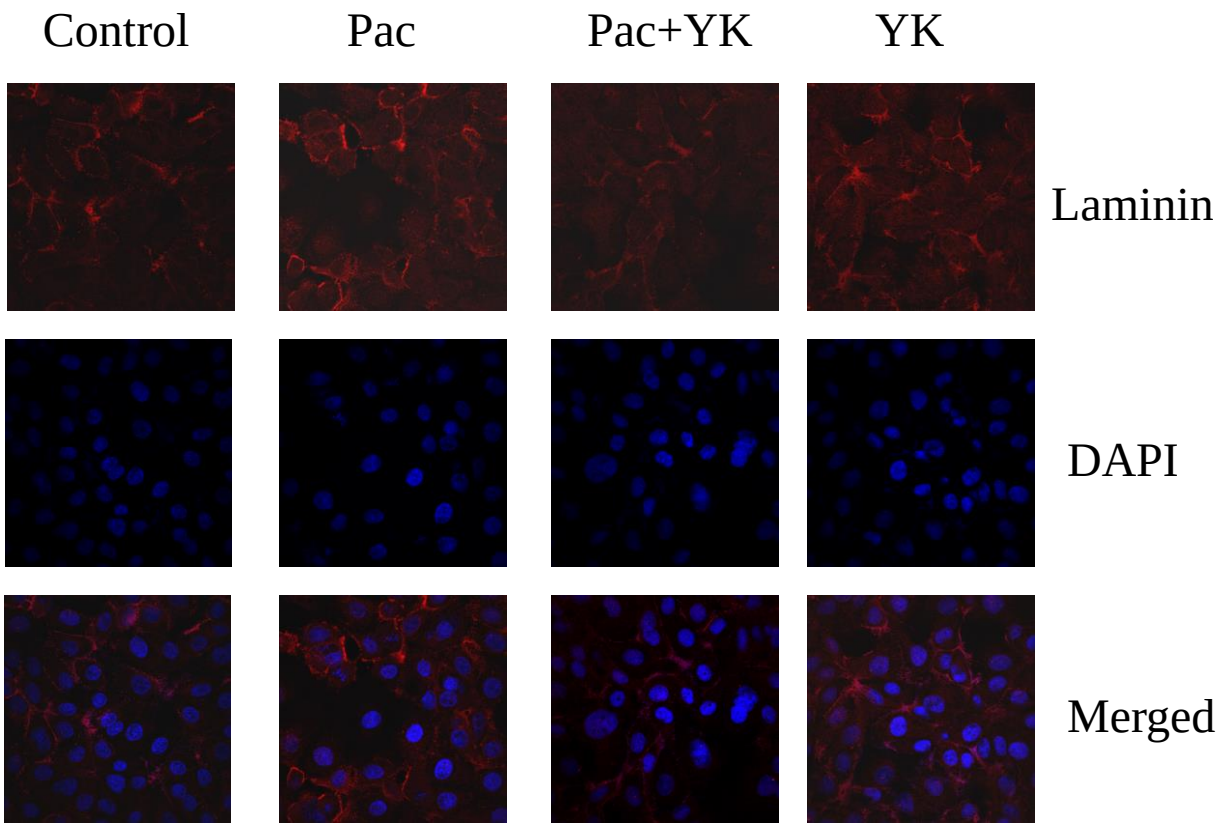
Level of ECM components- Caveolin

- 22-24 Kda integral membrane protein
- Down regulated of Cav 1 in cancer
- Negative regulatory role in tumor development
- Act as tumor suppressor
- Cav1 in association with E-cadherin mediates cell to cell adhesion

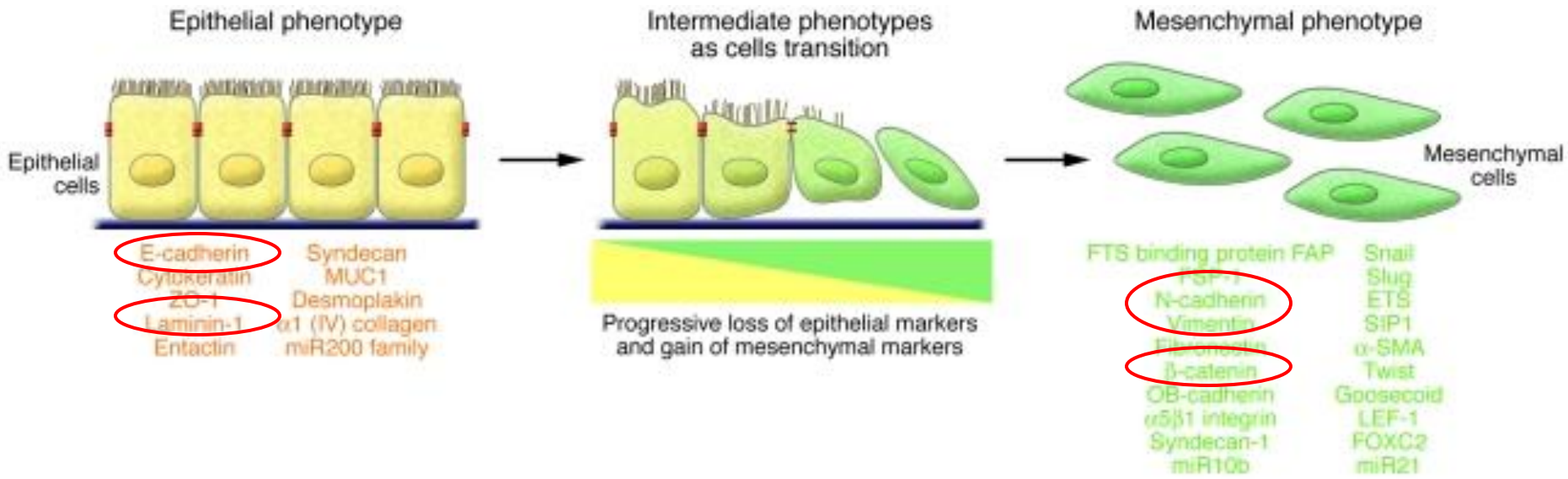




Immunofluoroscence distribution of Laminin (67kDa)



Epithelial to mesenchymal transition



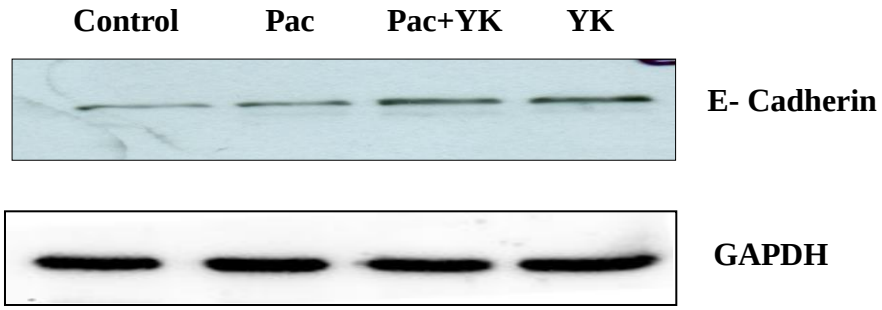
Progression towards malignancy is accompanied by

- Loss of epithelial differentiation
- Shift towards mesenchymal phenotype
- Characterized by invasive nature of cells
- Marked by increase in expression of mesenchymal marker and decrease in epithelial marker

Effect of *Yukyung Karne* on Epithelial markers

E-Cadherin:

- ✓Single span transmembrane 135kDa protein
- ✓Mediates cell – cell adhesion
- ✓Regulation of cell polarity and maintenance of epithelial organization
- ✓Loss of E-cadherin : predictive of poor survival



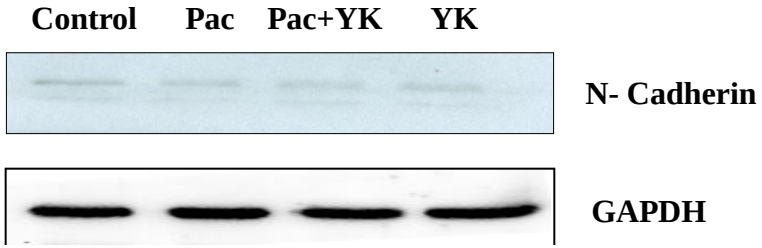
Effect of *Yukyung Karne* on mesenchymal markers

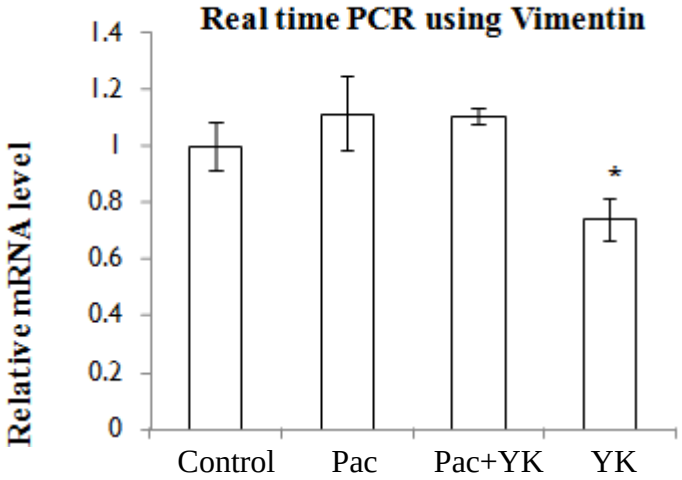
N-Cadherin

- Associated with acquisition of EMT phenotype

Vimentin

- Requisite regulator of mesenchymal cell migration





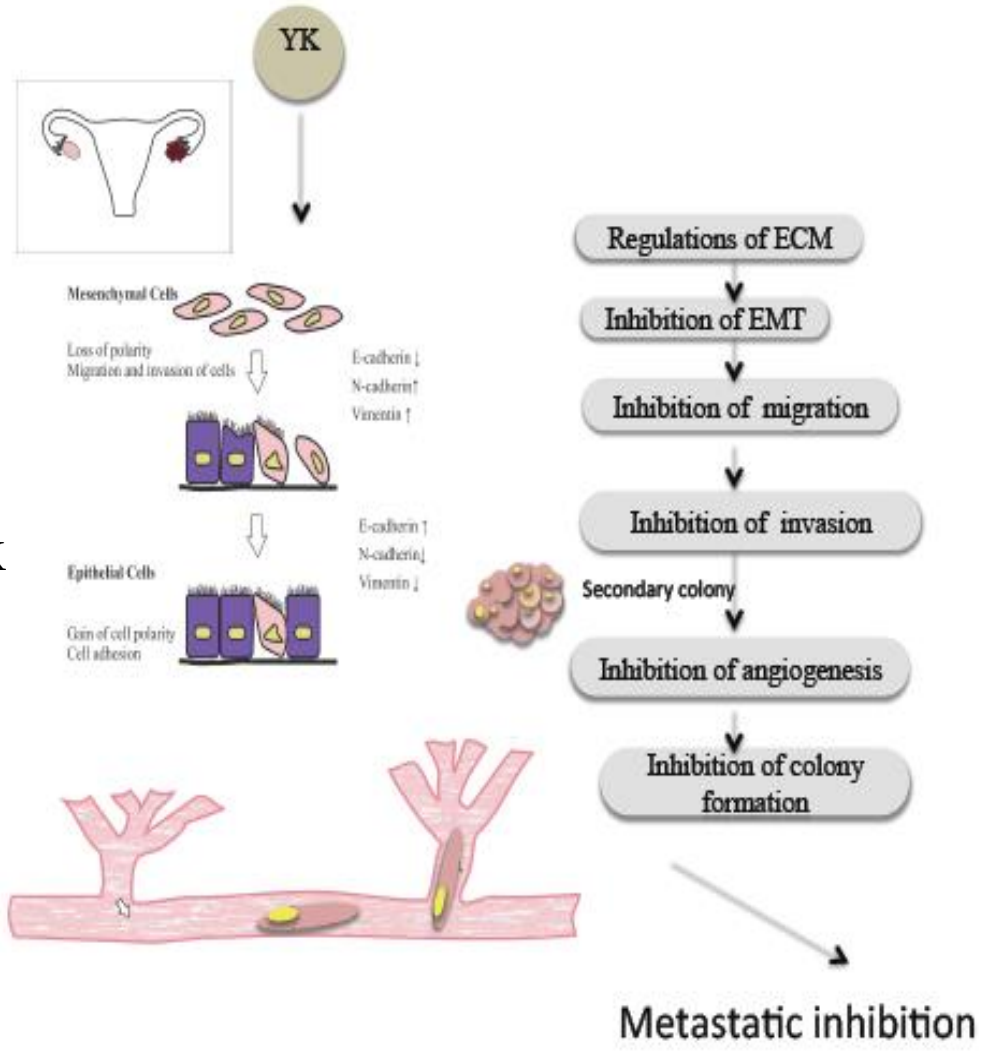
:*, P<0.05

Effect of *YK* on 5 major steps of metastasis

- Inhibits collagen mediated cell adhesion
- Inhibits active invasion and migration
- Suppresses the growth at secondary sites
- Inhibits VEGF secretion for angiogenesis

Effect of *YK* on ECM and EMT

- Repression of matrix metalloproteinases (MMP2/9)
- Effects major components of ECM-
- Down regulates β-Catenin,Integrin and pFAK
- Upregulates Caveolin and Laminin
- Regulates Epithelial to mesenchymal transition
- It increase in epithelial marker and decrease in mesenchymal marker



Conclusions

TTM-*Yukyung Karne* possesses strong anticancer property

- ✓*Yukyung Karne* induces selective apoptosis
- ✓Imposes G1 arrest
- ✓Restoration of tumor suppressor p53 and pTEN
- ✓Regulation of cell cycle regulator
- ✓Inhibits uncontrolled cell proliferation
- ✓Induces apoptosis via mitochondrial dependent intrinsic pathway
- ✓ It stimulates permeabilization of mitochondrial membrane and release of Cytochrome C

Choedon et al. BMC Complementary and Alternative Medicine 2014, 14:380
http://www.biomedcentral.com/1472-6882/14/380

BMC Complementary & Alternative Medicine

RESEARCH ARTICLE Open Access

Molecular insights into the anti-cancer properties of Traditional Tibetan medicine *Yukyung Karne*

Tenzin Choedon[1,2], Dawa Dolma[3], Ganeshan Mathan[2] and Vijay Kumar[1*]

Abstract

Background: *Yukyung karne (YK)* is a traditional Tibetan formulation used for many centuries for the treatment of ovarian cancer. However, the pharmacological basis of its anticancer property is not well understood. In the present study, the anticancer property of YK was investigated in cell culture.

Methods: The growth inhibitory property of *YK* was evaluated in SKOV6, IHH, HepG2 and HEK293 cell lines using MTT assay. The pro-apoptotic activity of drug was analyzed by terminal deoxynuleotidyl transferase dUTP nick end labeling (TUNEL) and DNA fragmentation assays. Confocal microscopy was used to show the release of cytochrome c and its co-localization with mitochondria with the help of dsRed mitotracker in SKOV6 cells. The inhibition in cell proliferation was also visualized by confocal microscopy after BrDU incorporation. The activation of tumor suppressor p53 was evaluated by Western blotting while VEGF levels in culture supernatant were measured by a colorimetric method.

Results: *YK* specifically and efficiently induced apoptotic killing of the human ovarian cancer SKOV6 cells as indicated by increased DNA fragmentation and nick end DNA labeling. Confocal microscopy suggested inhibition of cell proliferation and increase in cytochrome c release via perturbation in mitochondrial membrane potential ($\Delta\psi m$). Further, *YK* up-regulated the expression of tumor suppressor p53 and key cyclin-dependent kinase inhibitor p21, and inhibited VEGF secretion by cells. Interestingly, *YK* also exhibited a synergy with paclitaxel which is a well-known anti-cancer therapeutic drug.

Conclusions: The pharmacological properties of *YK* to impose growth arrest and trigger pro-apoptotic death in cells amply justify its usage in primary as well as adjunct therapy for ovarian cancer.

Keywords: *Yukyung Karne*, Traditional Tibetan medicine, Ovarian cancer, Apoptosis, Mitochondria membrane potential

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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 for the treatment of ovarian cancer. Though *Yukyung Karne* has been reported to be clinically effective, the molecular mechanism of its anti-metstatic 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

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