



H19: an example of lncRNA- mediated chemoresistance

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Colorectal Cancer and Chemotherapy

Colorectal cancer (CRC) is the third most prevalent cancer type and the third leading cause of cancer related deaths worldwide.

↓

Adjuvant chemotherapy has been demonstrated to extend life expectancy and improve the possibility of cure in patients with advanced colorectal cancer.

↓

However, most colorectal cancers remain **unresponsive to chemotherapy** and thus it is still a life-threatening disease and a big challenge to the clinics.

Drug resistance acquisition is one of the main issues in chemotherapy

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Intrinsic Chemoresistance

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Acquired Chemoresistance

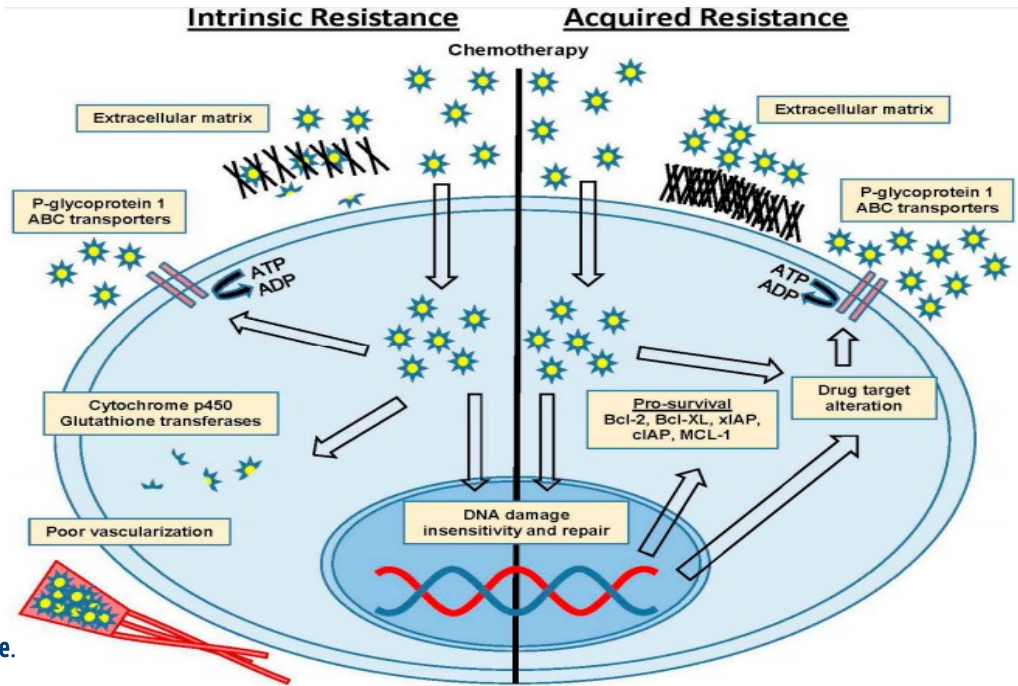
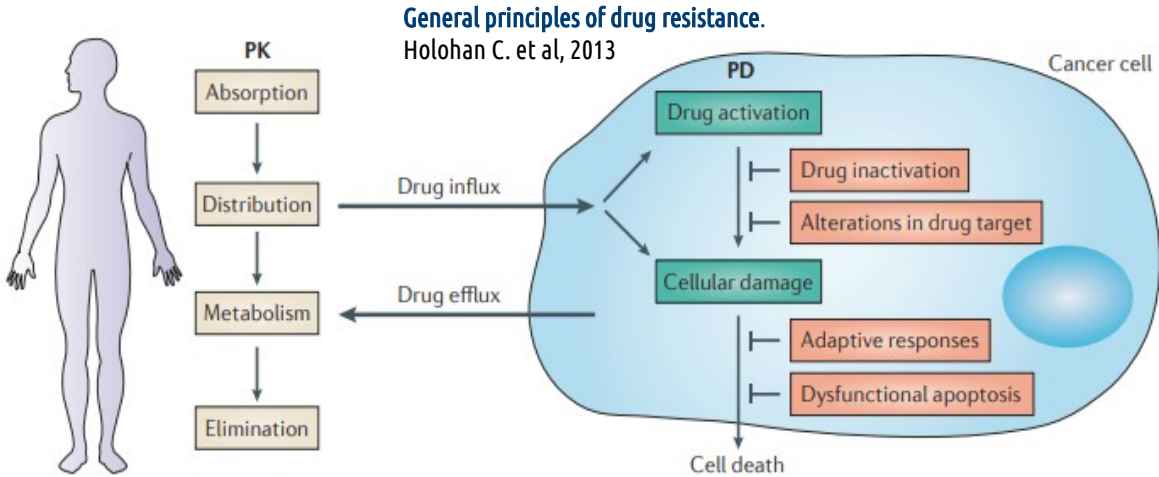
Cancer Chemoresistance

A diverse range of anatomic and molecular mechanisms have been implicated in drug resistance.

Pharmacokinetic Resistance

Physics of the tumor site

Pharmacodynamic Resistance



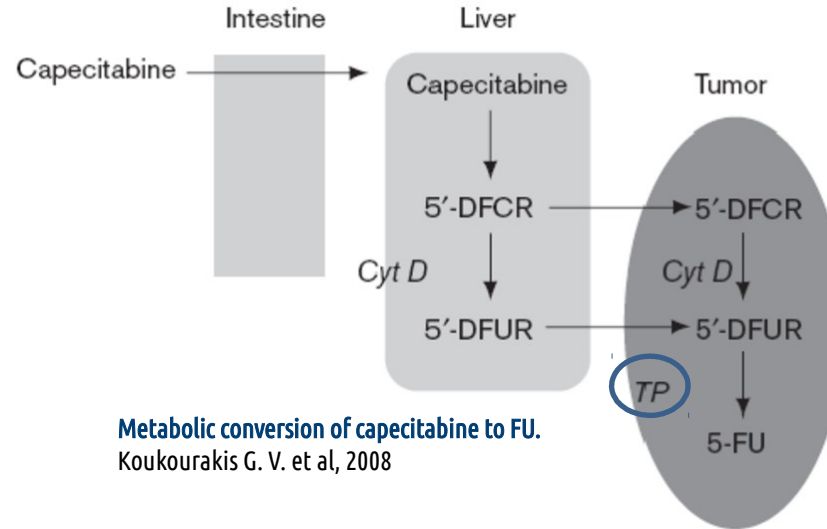
Intrinsic and Acquired chemoresistance.
Cornelison R. et al, 2017

Mechanisms of Chemoresistance

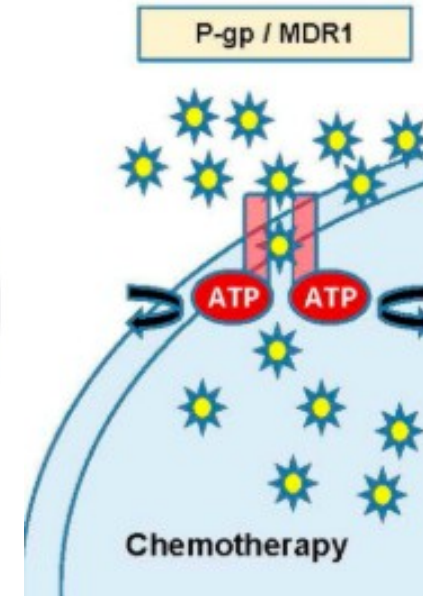
Drug transport and metabolism

Drug Efflux: Several cell membrane transporter proteins promoting drug efflux.

Drug activation and inactivation: drug inactivation or lack of activation.



Metabolic conversion of capecitabine to FU.
Koukourakis G. V. et al, 2008



MDR1 mediated chemoresistance.
Cornelison R. et al, 2017

Alterations in drug targets

Change in expression of Target: Genetic or epigenetic mutations that causing the over-expression of target molecules.

Change in binding domain of Target: Mutation in specific target molecules that don't block their activity.

Mechanisms of Chemoresistance

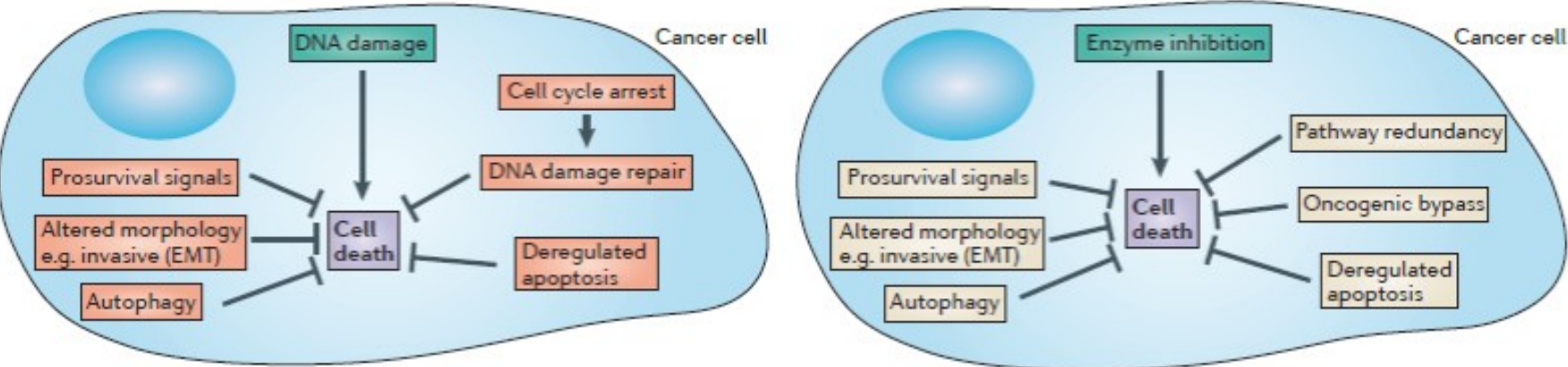
Dysfunction of DNA damage repair

Chemotherapeutic drugs induce DNA damage which may contribute to the acquisition of cytotoxicity. Cancer cells tend to be more resistant as a result of abnormal DNA damage by misregulation of DNA repair and DDR pathways.

Downstream resistance mechanisms

Downregulation of Apoptosis: Genetic/Epigenetic mutations causing overexpression of anti-apoptotic proteins or TFs that controls apoptotic pathways and downexpression of pro-apoptotic proteins.

Autophagy: Can facilitate cancer cell survival during metabolic stresses caused by anticancer agents.



Summary of downstream factors that influence drug resistance. Holohan C. et al, 2013

LncRNAs

LncRNA expression is widely altered in cancers and that lncRNAs participate in various aspects of tumorigenesis:

Cell proliferation, Apoptosis, Migration and Invasion, **Drug Resistance**.

What are LncRNAs?

LncRNAs include different types of RNA polymerase II (Pol II)- transcribed molecules with sizes **over 200 nt** in length.

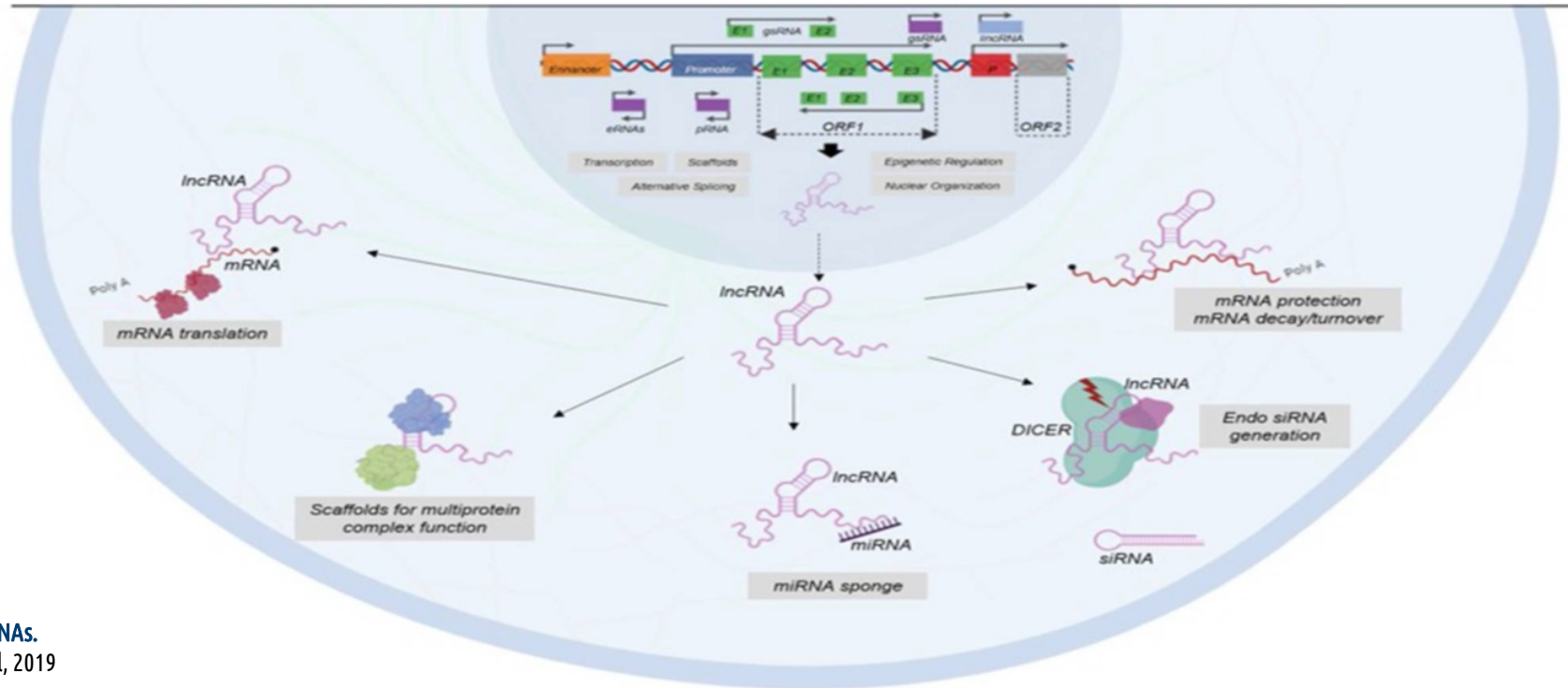
There are an estimated abundance of 5,400 to **more than 10,000 lncRNAs transcripts in humans**. They often harbor a poly-A tail and can be spliced, similar to mRNAs.

It has been shown that **lncRNAs functions depend on their subcellular location**: a wide range of subcellular localization patterns, including nucleus, cytoplasm and both.

LncRNAs: Functions

Roles in nuclear structures: they help to the structure of nuclear speckles, paraspeckles, and interchromatin granules.

Roles in regulation of gene expression at transcriptional and post-transcriptional levels in multiple biological processes and cellular contexts.



LncRNAs in Drug resistance

LncRNA	Function
GIHCG	Potential target in 5-FU and Oxaliplatin resistance mechanisms.
MIR100HG	Coordinately MIR100HG, miR-100 and miR-125b overexpression drives Cetuximab resistance by targeting five negative regulators of Wnt signaling which have a potential clinical relevant interaction with EGFR.
UCA1	UCA1 can decrease the sensitivity of CRC cells to 5-FU by sponging miR-204-5p resulting in attenuating apoptosis. Moreover, UCA1 expression levels are increased in Cetuximab resistant cells and can be transferred to sensitive cells through exosomes increasing resistant cells number.
LINC00152	LINC00152 confers Oxa and 5-FU chemoresistance by sponging miR-193a-3p by ERBB4 modulation and then inducing the activation of AKT signaling pathway that mediates cell survival and chemoresistance. miR-193a-3p also targets NOTCH1 regulating CRC growth, metastasis, stemness, and chemoresistance.
HOTAIR	HOTAIR could regulate the progression and Cisplatin and Paclitaxel chemoresistance enhancements in CRC by targeting miR-203a-3p and the activity of Wnt/ β -catenin signaling pathway.
PCAT-1	PCAT-1 regulates the invasiveness and 5-FU resistance in CRC cells and that PCAT-1 may promote CRC cell invasion by modulating the expression of c-Myc.
PVT1	PVT1 is associated with 5-FU resistance in human CRC tissues and cells by inhibiting apoptosis and upregulating the expression of MRP1, P-gp, mTOR, and Bcl-2
XIST	XIST promotes Doxorubicin resistance through sponging miR-124 which targets SGK1 increasing cell survival, loss of control in cell cycle, inhibiting apoptosis, and increasing chemoresistance.
MALAT1	Overexpression of MALAT1 enhances chemoresistance in 5-FU resistant cells through potentiation of multidrug resistant genes such as MDR1, MRP1, BCRP, and ABC. Moreover, modulates EZH2 pathway in Oxa resistance
H19	H19 mediated Methotrexate resistance via activating Wnt/ β -catenin signaling, which help to develop H19 as a promising therapeutic target for MTX resistant CRC. Besides, CAFs promote stemness and Oxa chemoresistance in CRC by transferring exosomal H19 to CRC sensitive cells through sponging miR-141.
SLC25A25-AS1	SLC25A25-AS1 has a pivotal role in CRC cells promoting chemo sensitivity to 5-FU and DOX via Erk and p38 pathway modulation. Hence, SLC25A25-AS1 was determined to play a tumor suppressive role in CRC.
snar	snar has a negative regulator role in responsible of the development of 5-FU resistance through cell growth of CRC cells. Nonetheless, snar detailed roles have not yet been established.
ENST00000547547	ENST00000547547 reduced the chemoresistance of 5-FU via competitive sponging to miR-31 which targets ABCB9 involved in chemotherapy induced apoptosis. This suggests that lncRNA ENST00000547547 may be a positive prognostic factor for 5-FU-based chemotherapy.
TUG1	TUG1 mediates MTX resistance in colorectal cancer via sponging miR-186 that targets CPEB2 increasing its protein levels that play an important role in tumorigenesis and chemoresistance.
PVT1	PVT1 is a significant regulator in tumorigenesis and cisplatin resistance of CRC by inhibiting apoptotic pathways in CRC and may serve as a promising target for CRC therapy.
MEG3	MEG3 promotes chemosensitivity to Oxa by inducing cytotoxicity in CRC cells promoting apoptosis. In addition, MEG3 sponges miR-141 that targets PDCC4.

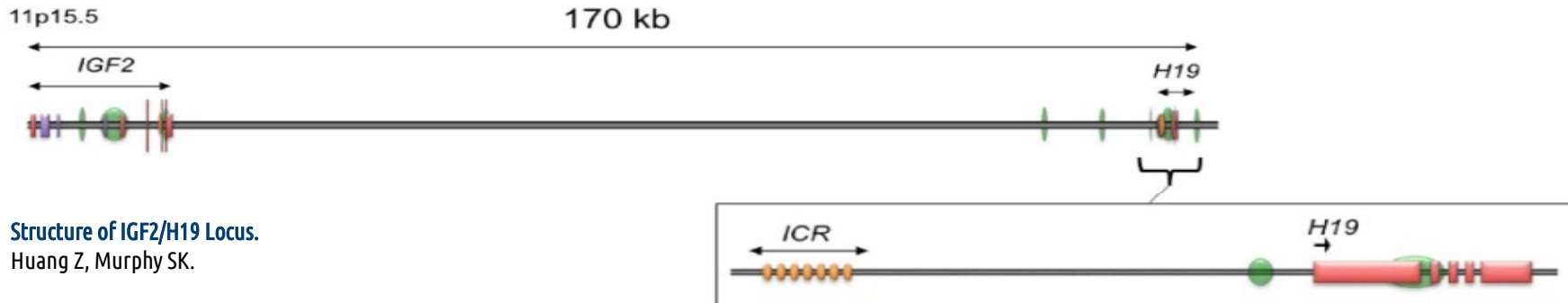
LncRNAs are involved in cancer cell drug resistance with different regulated targets/pathways

LncRNA H19

H19 gene is located in 11p15.5 downstream the **insulin-like growth factor 2 (Igf2)** gene and encodes for a 2,3kb capped, spliced and polyadenylated RNA.

H19 is highly expressed from the onset of embryogenesis to fetal life in vital organs such as the fetal adrenal, liver, and placenta but is downregulated in postnatal stages.

Plays pivotal roles in embryonal development and growth regulation



Structure of IGF2/H19 Locus.
Huang Z, Murphy SK.

Imprinting regulation of H19 Expression

Located in an imprinted region of chromosome 11.

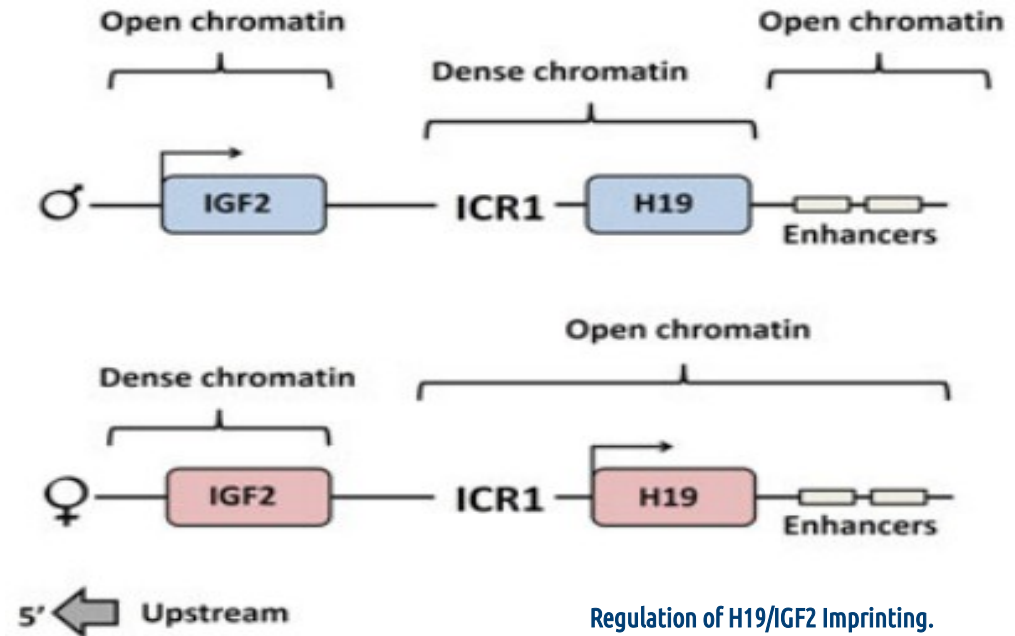
H19 and **Igf2** are oppositely imprinted and co-expressed embryonic development suggesting a common mechanism of regulation.

Important regulation elements

ICR1 is important to the imprinting state of both H19 and Igf2 and is rich in CpG residues.

Two downstream **enhancers** where open in chromatin structure on both parental alleles and could work on either Igf2 or H19.

CAGCCC motifs in ICR1 that binds **CTCF proteins**.



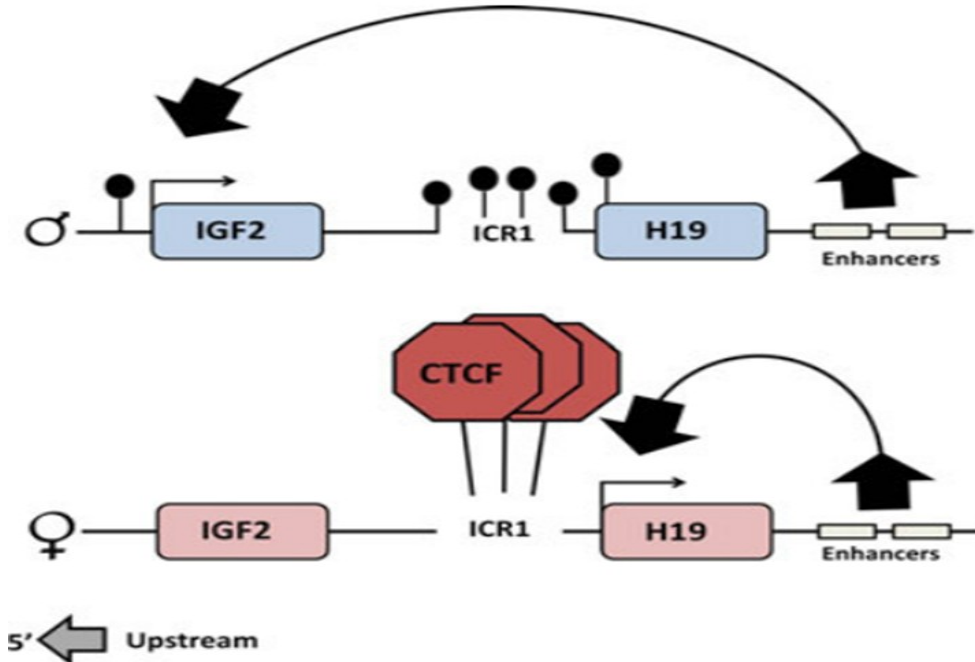
Imprinting regulation of H19 Expression

Enhancers could work on either IGF2 or H19 but:

The methylation of the paternal H19 by **DNMT** inhibits his expression and thus gives enhancers a chance to work on the IGF2 gene.

ICR1 and the H19 promoter are unmethylated on the maternal chromosome, leading to transcription of maternal H19.

The binding of **CTCF** to CAGCC unmethylated motif in ICR1 in maternal chromosome prevent downstream enhancers from accessing IGF2.



Regulation of H19/IGF2 Imprinting.
Nordin M et al, 2014

LncRNA H19 in Cancer

H19 can act as either an **oncogene** or **tumour suppressor**: the role of H19 may differ at different times in life, or display a cell dependent and/or tumour type-dependent function.

H19 is **upregulated** in several cancers as, esophageal cancer, hepatocellular carcinoma, ovarian cancer, bladder cancer, breast cancer and **colorectal cancer**.

It is implicated in cancer progression processes such as metastasis (enhances EMT), proliferation and drug resistance. In particular in CRC it can led to **Methotrexate** or **5-Fu** resistance.

H19 is the lncRNA with the most substantial correlation to CRC patient survival, serving as an independent predictor for OS and disease-free survival (DFS).

This lncRNA has been related with poor prognosis

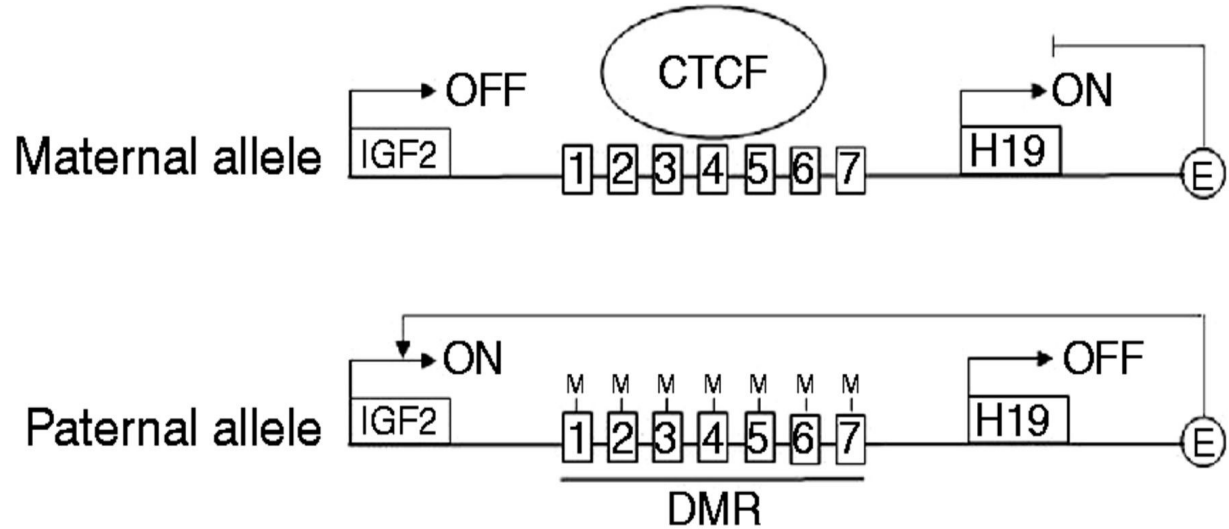
Loss of imprinting and cancer

We find **LOI** in many types of cancer.

There are evidence that **H19** is upregulated in many types of cancer.

In several types of cancer we can found **LOI** as the cause of the overexpression of **H19**.

In colorectal cancer we don't found **LOI** in **H19** ICR, however is overexpressed.

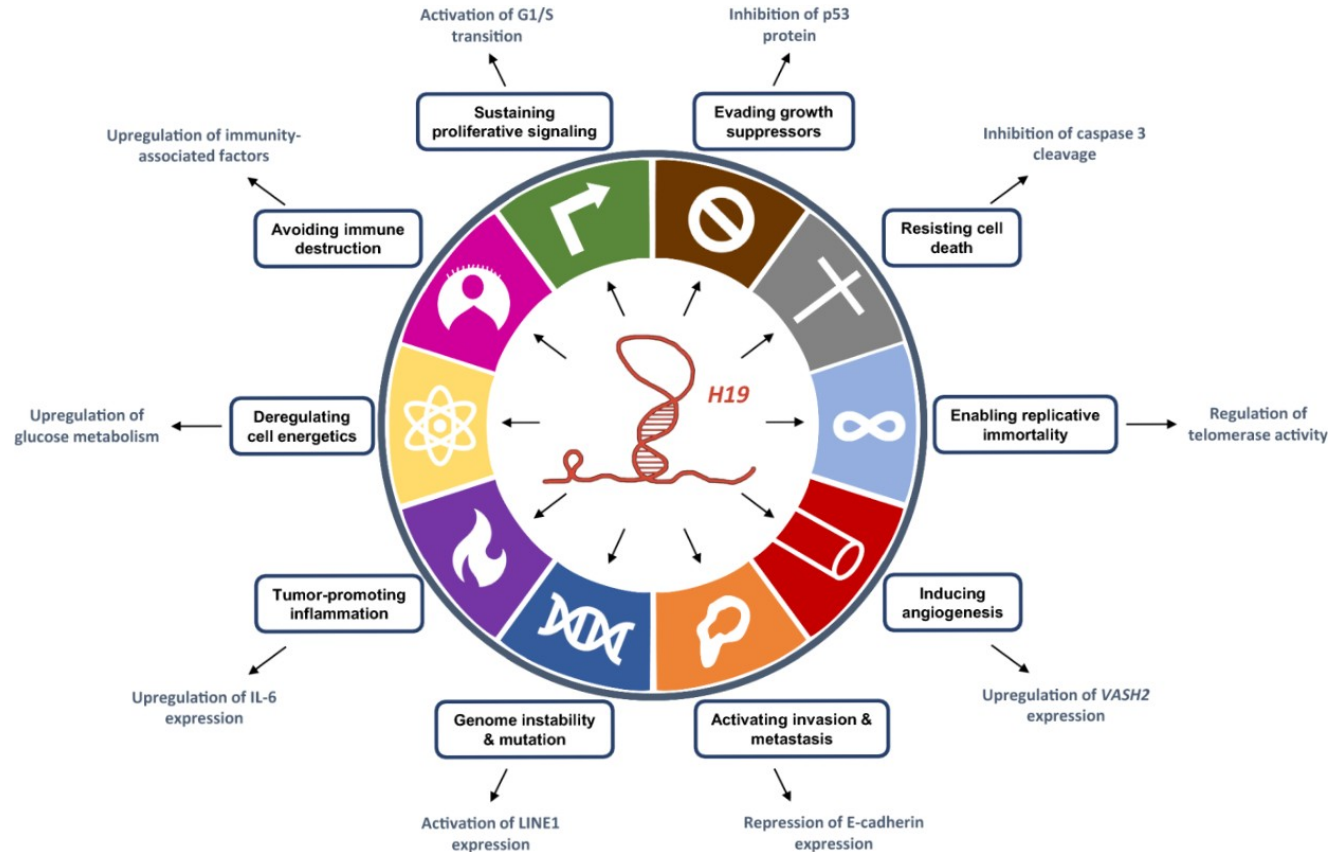


H19 expression in tumors influences several hallmarks of cancer

Promotes epithelial to mesenchymal transition in colorectal cancer via **Wnt/ β -catenin** pathway.

Promotes the migration and invasion of colon cancer cells via **MAPK** signaling pathway.

Promotes chemoresistance against several chemotherapeutics like **methotrexate** and **5-FU**.

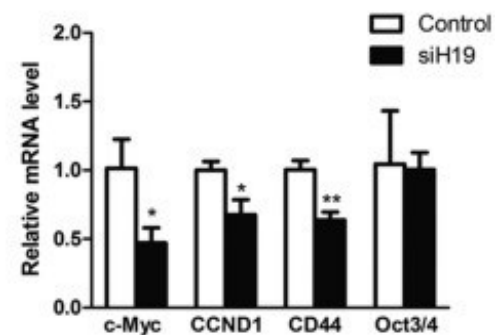
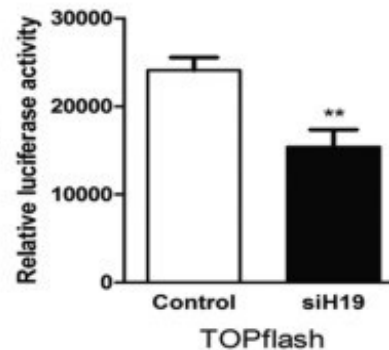
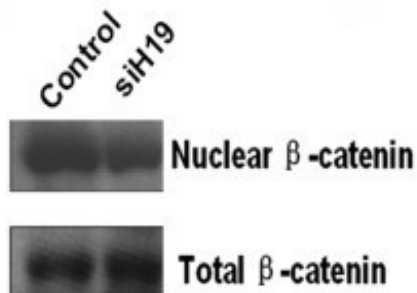
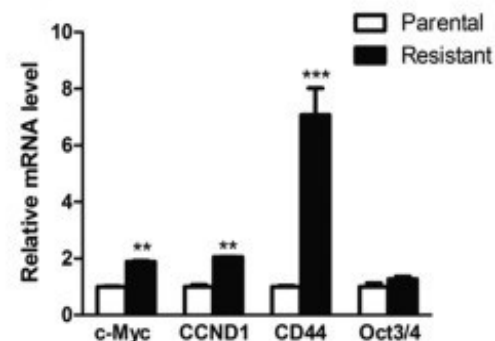
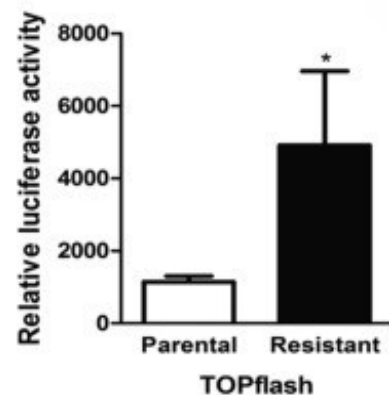
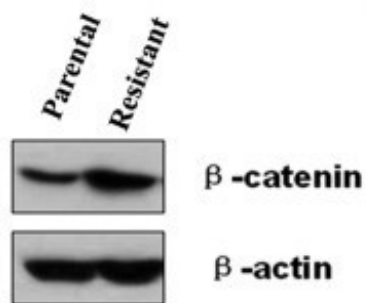


Chemoresistance mediated by H19 in tumors

H19 mediates methotrexate chemoresistance via **Wnt/ β -catenin** pathway.

Methotrexate inhibits dihydrofolate reductase (DHFR).

Knockdown of H19 improves the sensitivity to methotrexate of resistant cells.



Does H19 plays a role in colorectal cancer 5-Fu chemoresistance?

Often colorectal cancer is resistant to 5-Fu therapy.

Is necessary to find new targets for treatment of CRC 5-Fu resistant cases.

Therapy-induced autophagy is a mechanism of resistance to anticancer agents.

H19 promotes 5-Fu resistance in other types of tumor.

Wang et al. *Cell Death and Disease* (2018)9:1149
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Cell Death & Disease

ARTICLE

Open Access

Long non-coding RNA H19 confers 5-Fu resistance in colorectal cancer by promoting SIRT1-mediated autophagy

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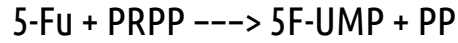
5-Fu based chemotherapy

5-Fu is a nucleoside analog inhibitor. Is a pyrimidine analog.

It blocks **deoxythymidylate** synthesis acting as a competitive inhibitor in **thymidylate synthase**.

It is used in chemotherapy for many types of cancer.

Often colorectal cancer is resistant to **5-Fu** therapy.



Uracil phosphoribosyltransferase (it also activates 5-Fu)

Next 5F-Ump must be converted to fluorodeoxyuridilate



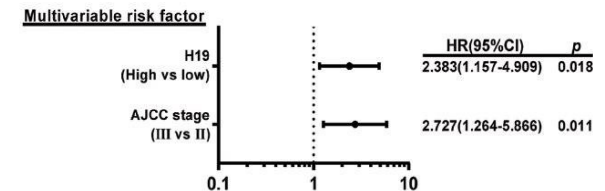
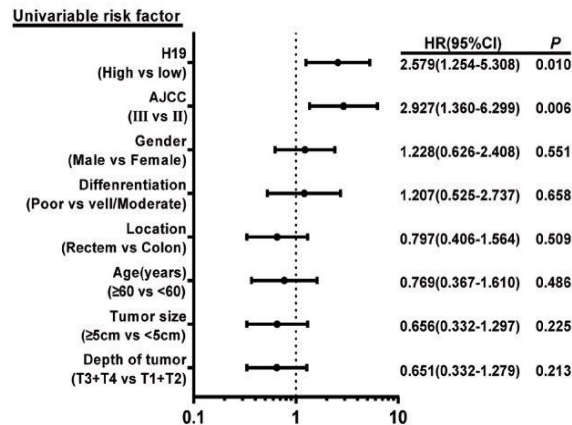
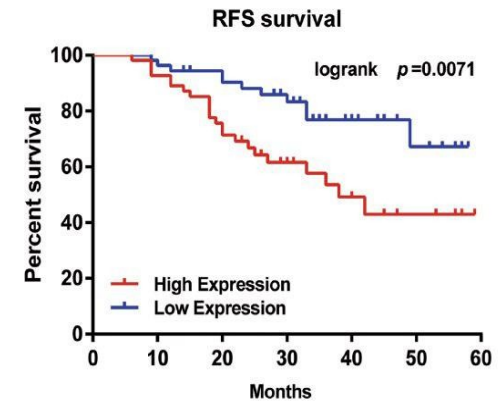
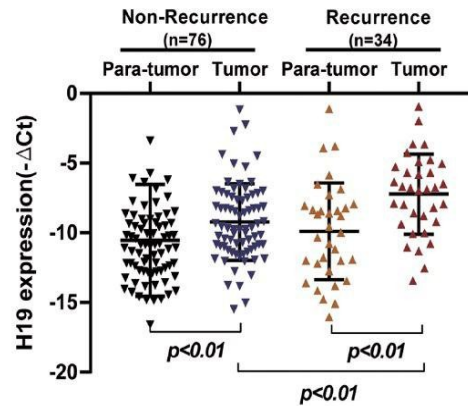
H19 is correlated with colorectal cancer recurrence

H19 indicates a poor prognosis of colorectal cancer.

H19 is an independent prognosis factor for CRC aggressiveness.

Expression of H19 could be significant to predict a clinical outcome.

H19 play a role in colorectal cancer recurrence



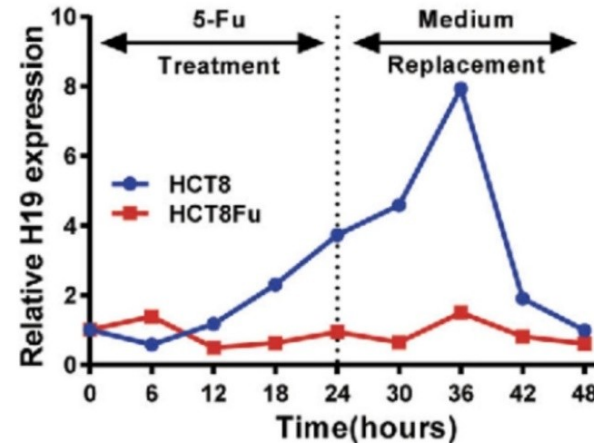
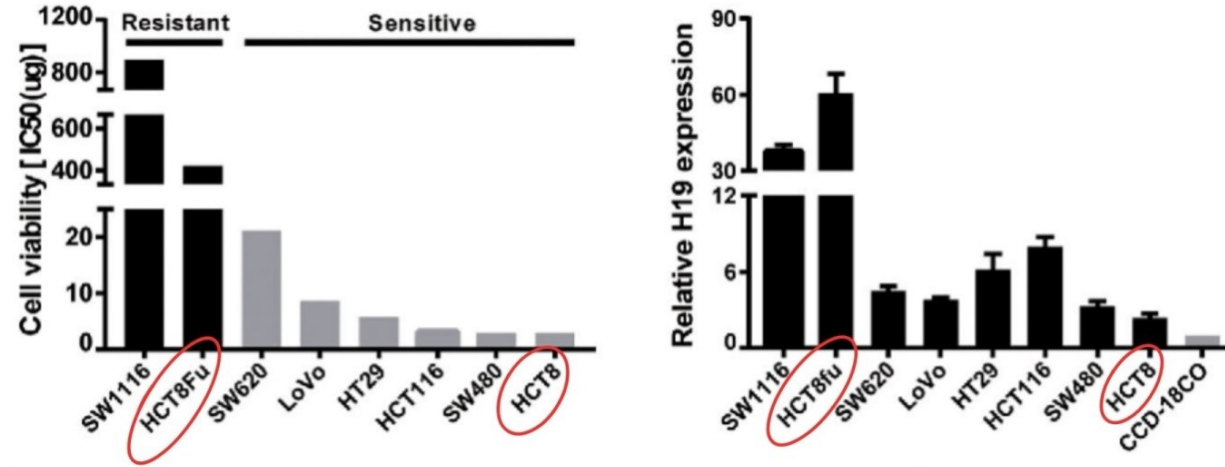
H19 increases the cells chemoresistance to 5-Fu

Creation and identification of cell lines 5-Fu resistant.

H19 is more expressed in cell lines 5-Fu resistant.

In drug sensitive cells, H19 is induced by treatment with 5-Fu.

Expression of H19 is correlated with cells 5-Fu chemoresistance

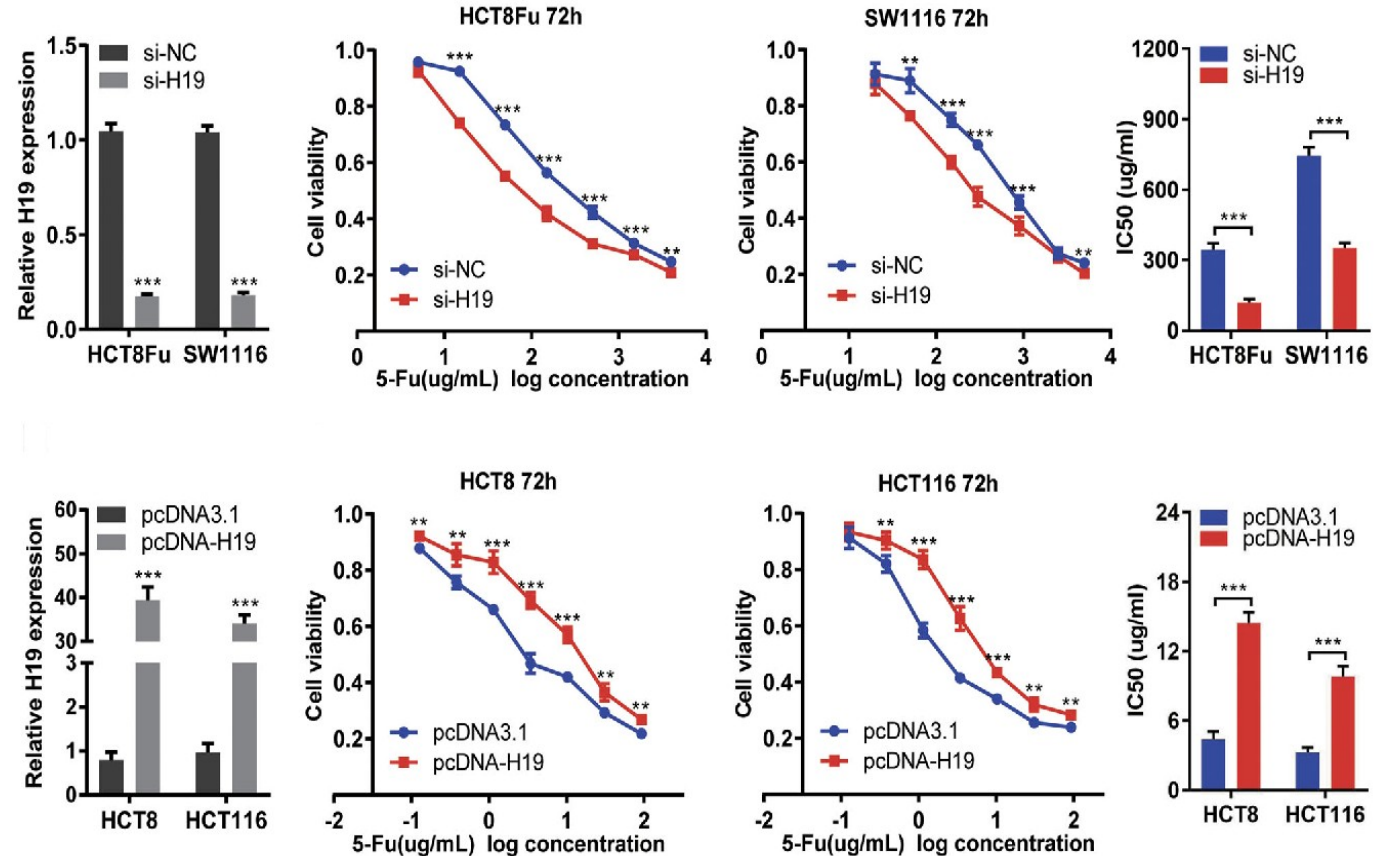


H19 plays a pivotal role in the chemoresistance to 5-Fu

Overexpression and knockdown of H19 was performed to see the effect on 5-Fu chemoresistance.

H19 increases IC50 value of 5-Fu in cell cultures.

H19 permits cells to survive in higher concentration of 5-Fu

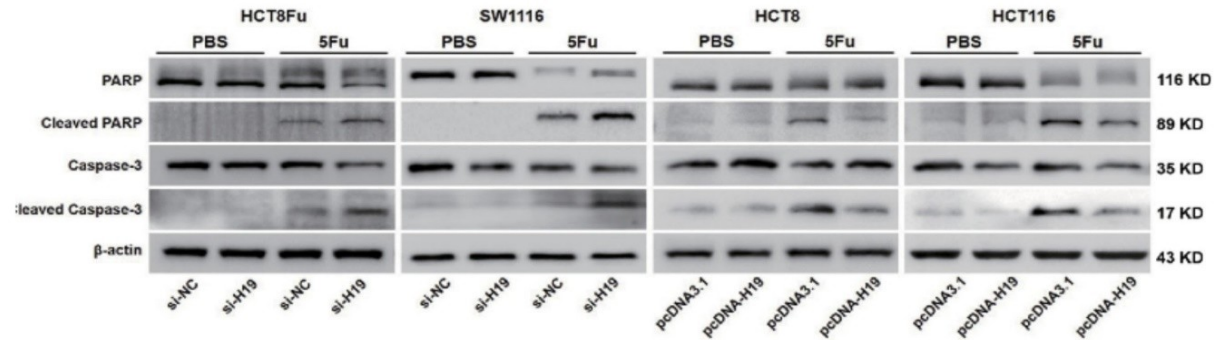
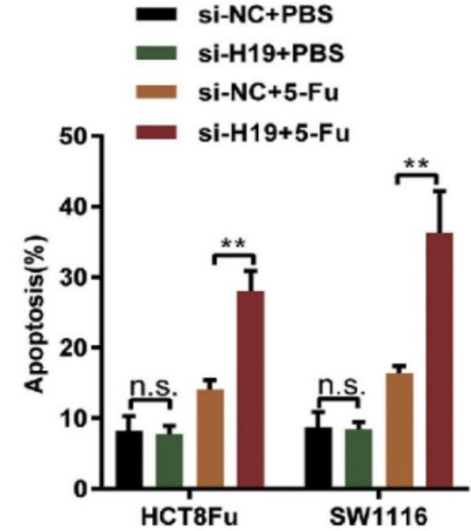
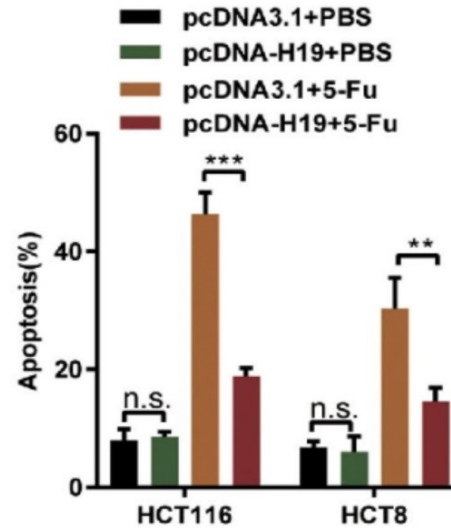


H19 influences apoptosis in presence of 5-Fu

Knock down H19 cell lines increases cell apoptosis when treated with 5-Fu.

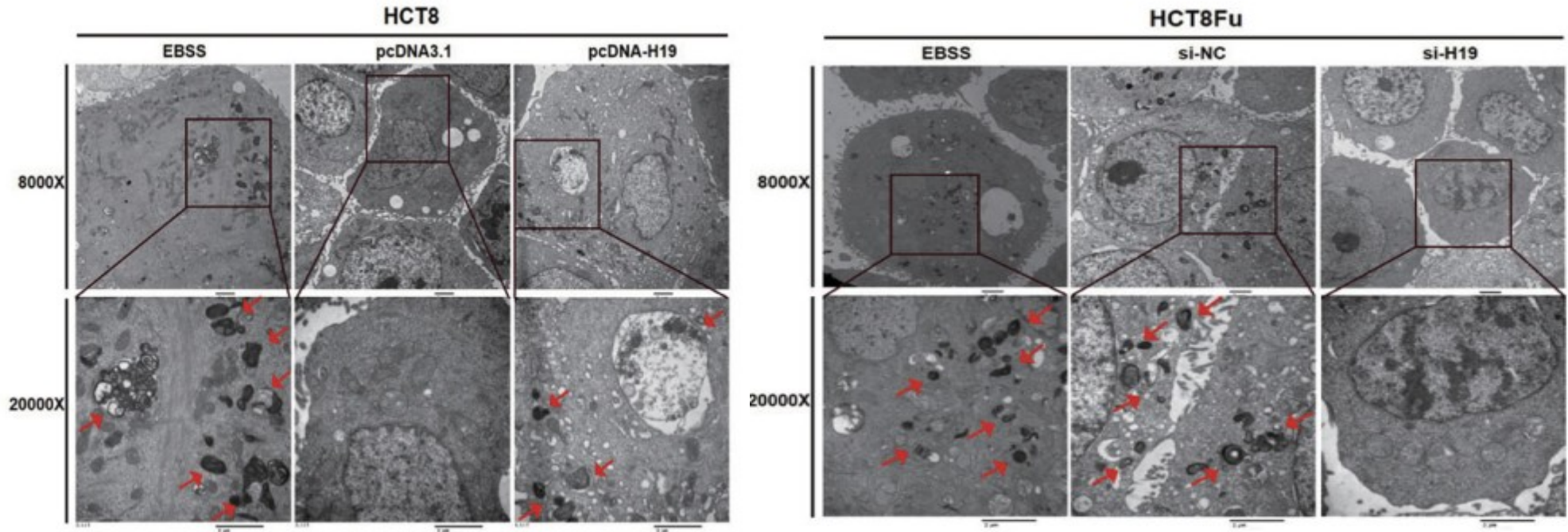
After treatment with 5-Fu levels of cleaved PARP and Caspase-3 increases.

H19 enhances resistance to 5-Fu via reducing cell death only under 5-Fu stress



H19 induces cancer chemoresistance via the autophagy pathway

Transmission electron microscopy showed an **increase** in the formation of **autophagic vesicles** in the **H19 overexpressed HCT8 cells**, and **lesser autophagic vesicles** in the **H19 knocked down HCT8Fu cells**

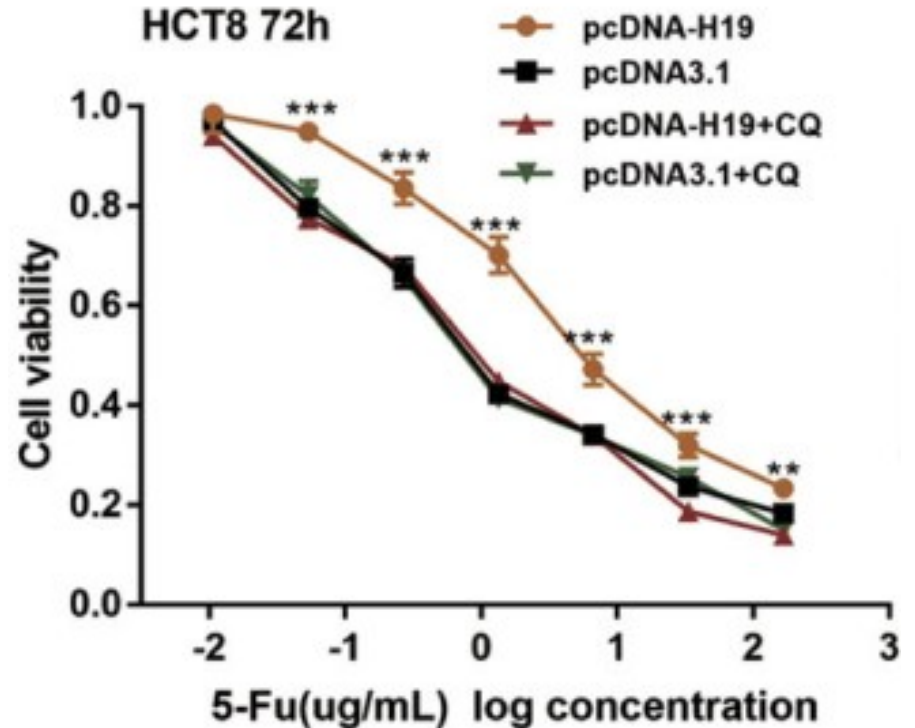


H19 induces cancer chemoresistance via the autophagy pathway

Addition of CQ could block the autophagic flux in the cells.

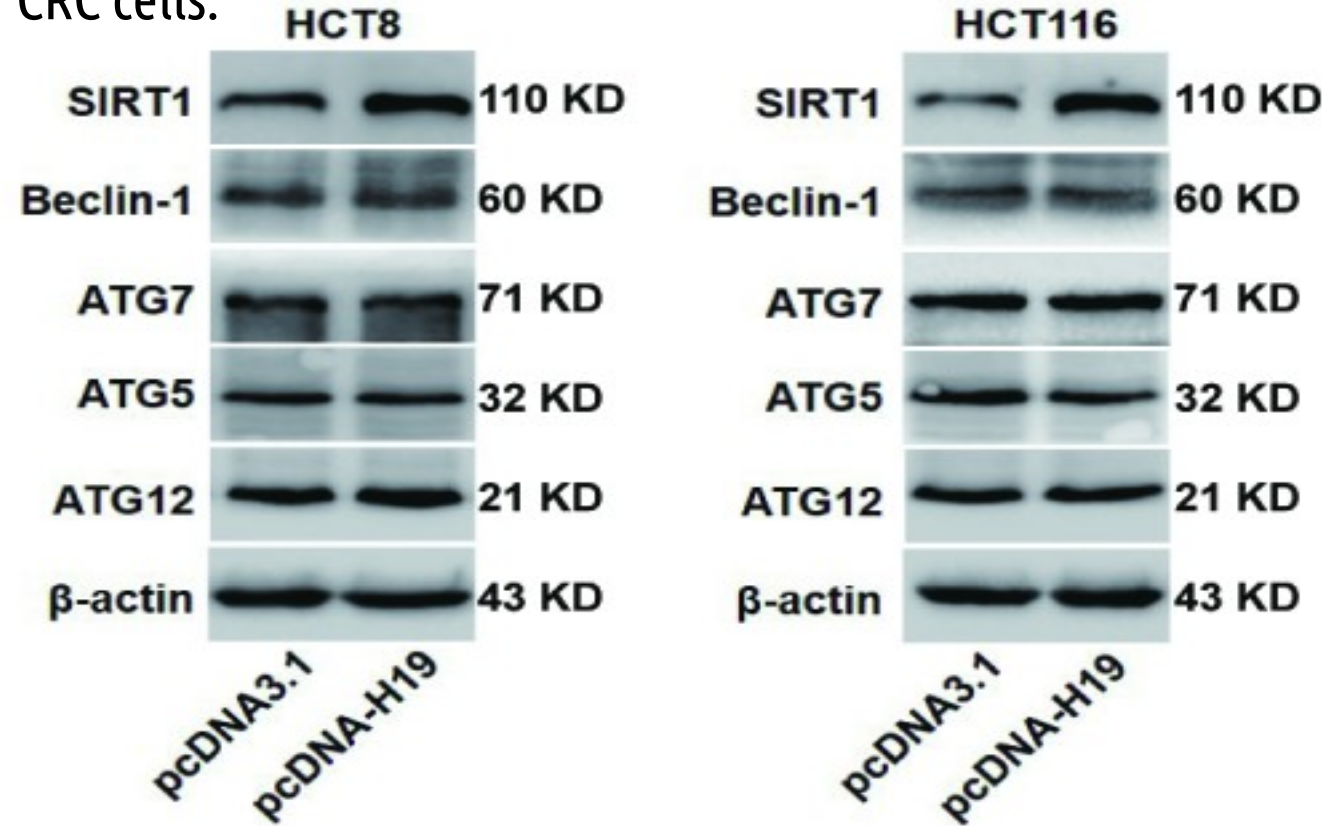
H19-induced 5-Fu resistance was abolished by CQ, which was confirmed by:

MTT assay



H19 triggers autophagy via SIRT1

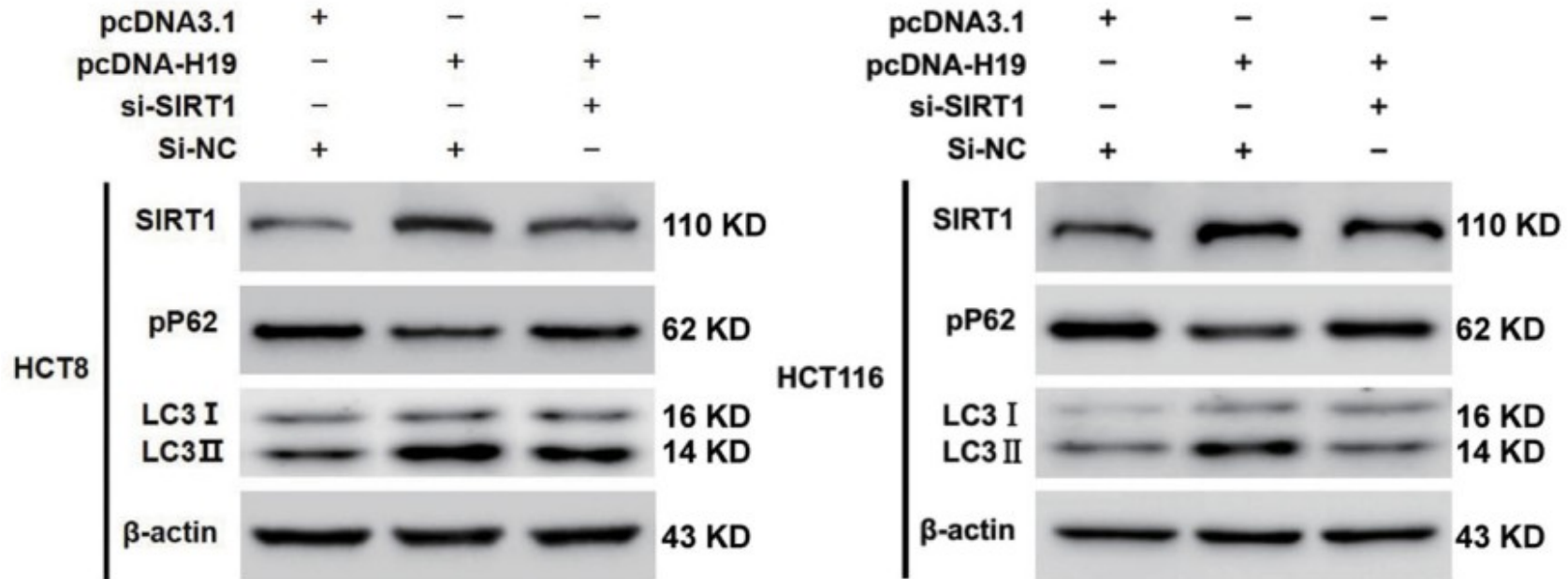
Western blotting was performed to analyze SIRT1 expression status in pcDNA-H19 transfected CRC cells.



Results showed that **only the expression of SIRT1 was upregulated after pcDNA-H19 transfected** both in HCT8 and HCT116 cells

H19 triggers autophagy via SIRT1

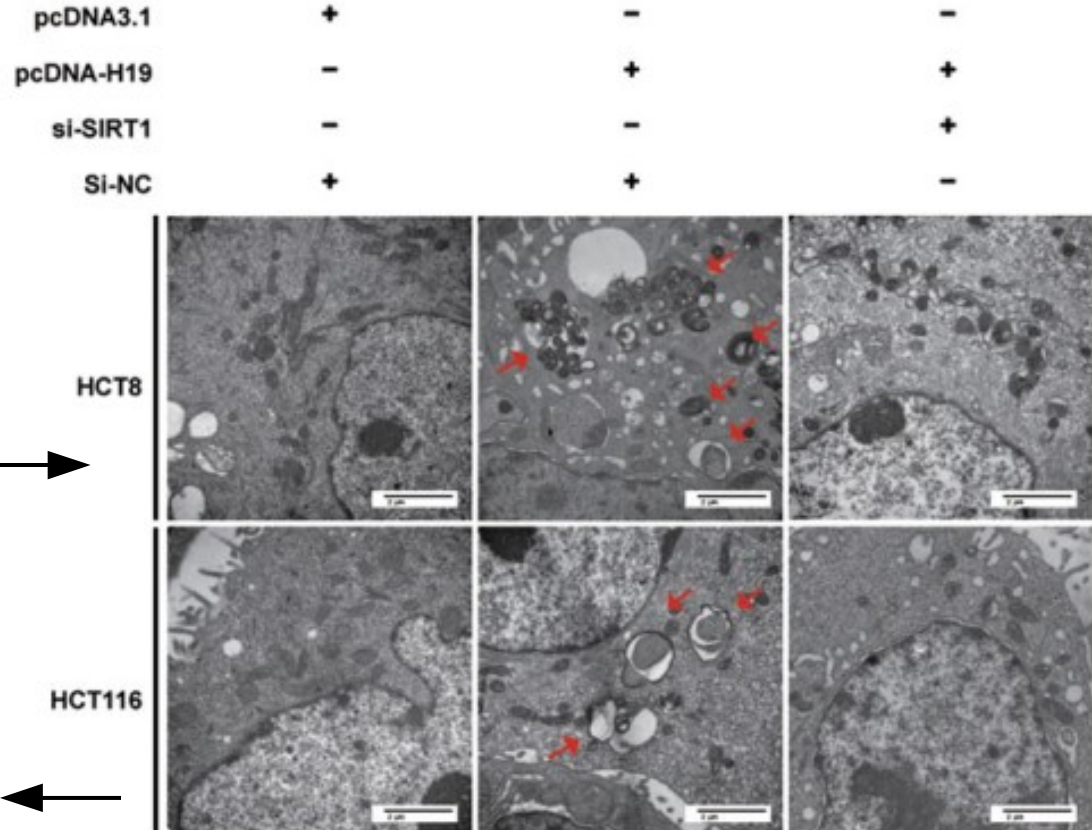
Western blotting was used to detect LC-3I, LC-3II, p62, and SIRT1 expression in HCT8 and HCT116 cells transfected with pcDNA-H19 and/or si-SIRT1



H19 triggers autophagy via SIRT1

Overexpression of H19 dramatically induced LC3 aggregation and increased the autophagosomes in CRC cells, which was markedly attenuated by the silence of SIRT1

All the above data indicated that H19 induces autophagy of CRC cells via upregulating the expression of SIRT1

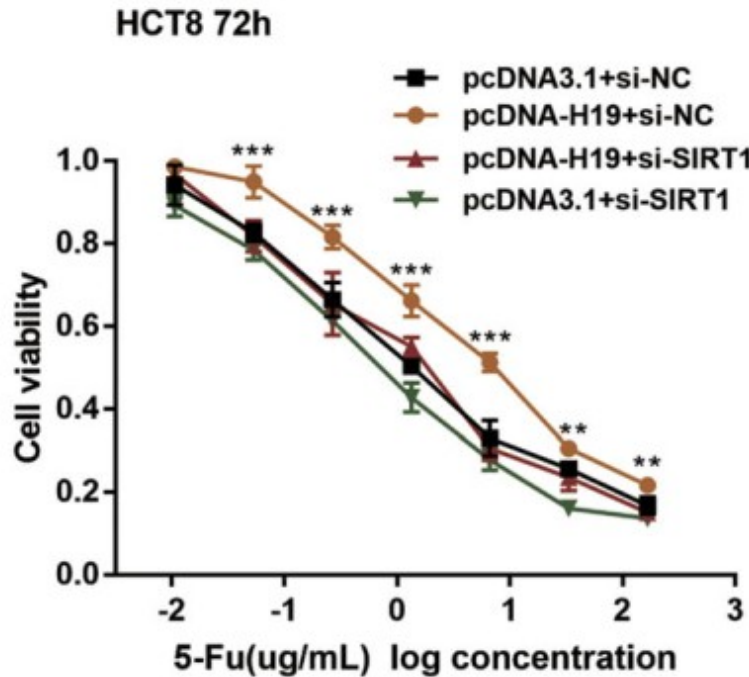


transmission electron microscopy

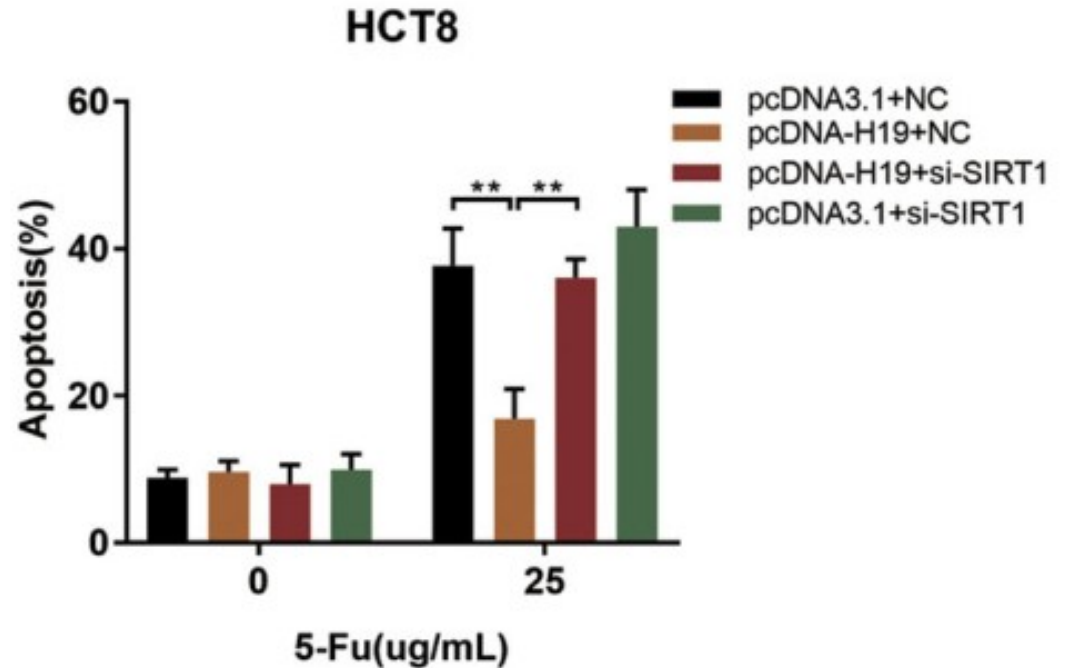
H19 triggers autophagy via SIRT1

Effects of SIRT1 on H19-induced 5-Fu resistance

observed that **SIRT1 knockdown** blocked the **H19-induced CRC cell 5-Fu resistance**



Cell sensitivity evaluated by MTT assay

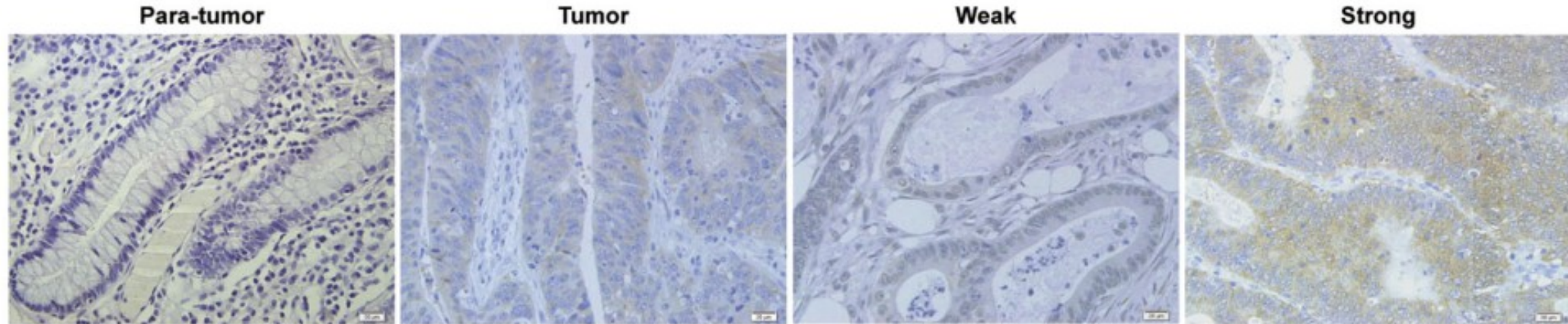


Apoptosis detected by flow cytometry

H19 triggers autophagy via SIRT1

The **expression of SIRT1** was detected by **immunohistochemical analysis**.

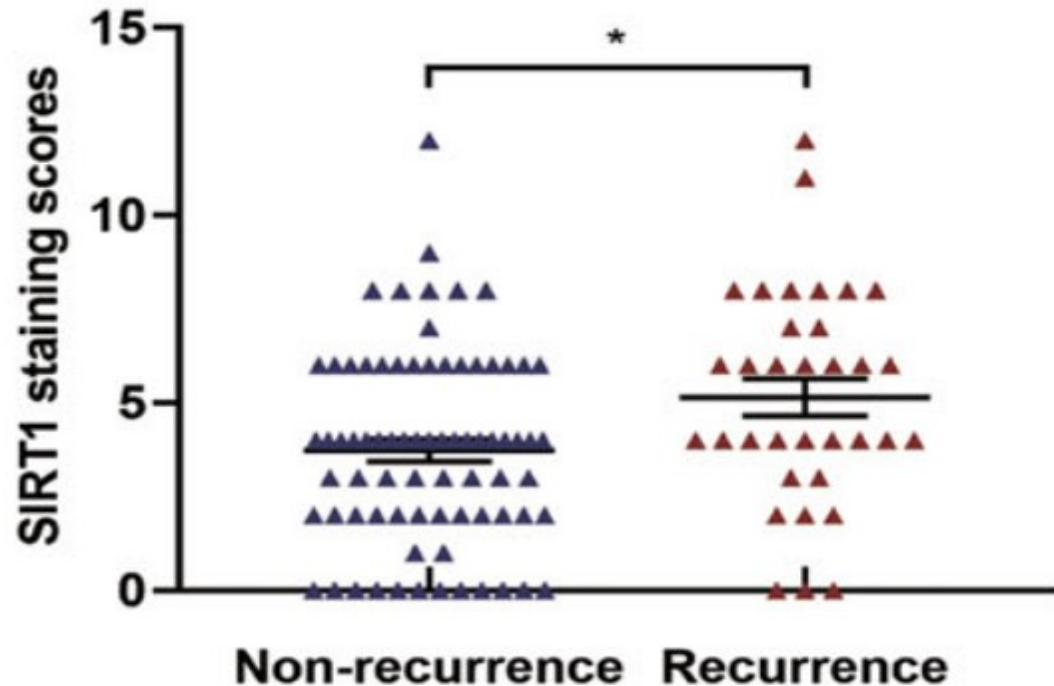
Results showed that **SIRT1** protein was **located in both cytosol and nuclei** of CRC cells



Expression of SIRT1 was analyzed by IHC in the CRC tissues and paired adjacent normal samples

H19 triggers autophagy via SIRT1

Recurrent CRC tissues from patients with recurrence had stronger SIRT1 expression compared with that of non-recurrence CRC tumor tissues.

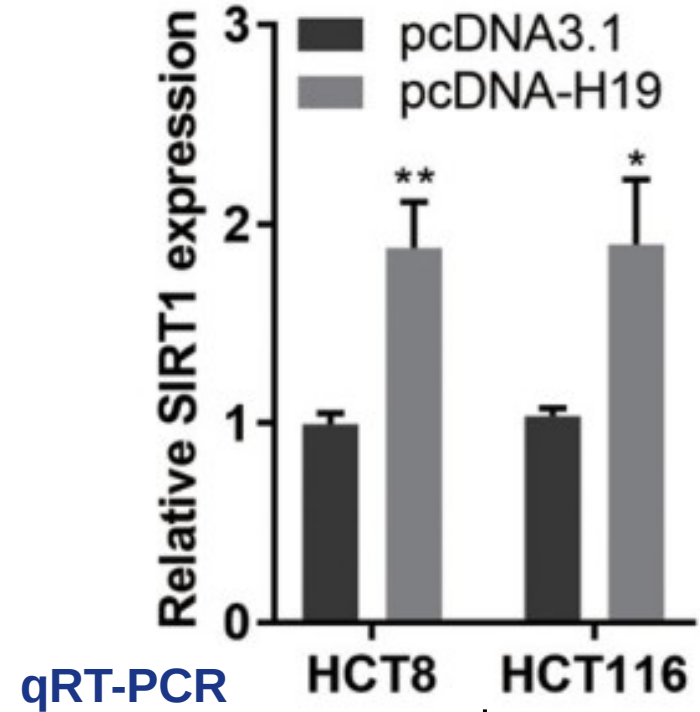
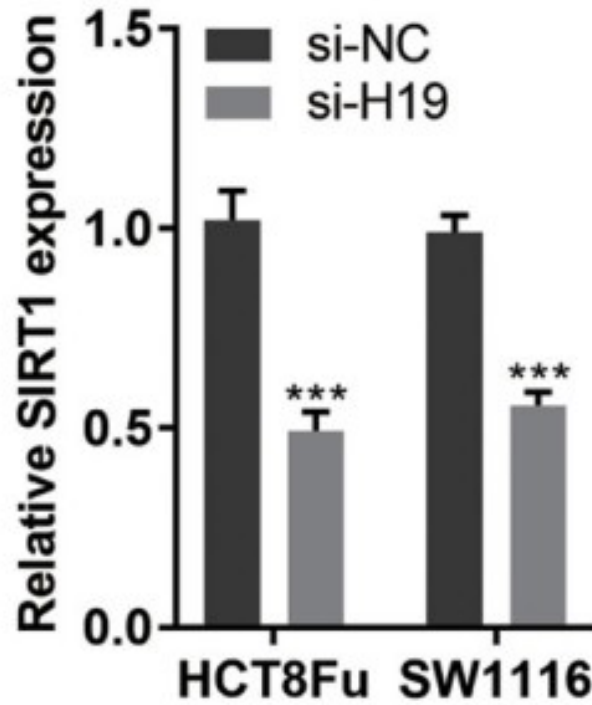


This phenomenon indicated that SIRT1 had underlying association with 5-Fu chemoresistance in CRC

IHC analysis was performed to determine the SIRT1 staining scores in CRC tissues with distinct recurrence status.

H19 triggers autophagy via SIRT1

To determine whether SIRT1 was a potential target gene of H19, they transfected cells with **pcDNA-H19** or **si-H19** and then **detected the expression of SIRT1**



H19 overexpression led to SIRT1 mRNA and protein levels increase at 72 h post transfection, and vice versa.

H19 sponges miR-194-5p as ceRNA

H19 is included in micro-RNA ribonucleoprotein complex (miRNP), probably through binding with miR-194-5p



Luciferase and RIP analysis confirmed the binding of H19 to miR-194-5p.



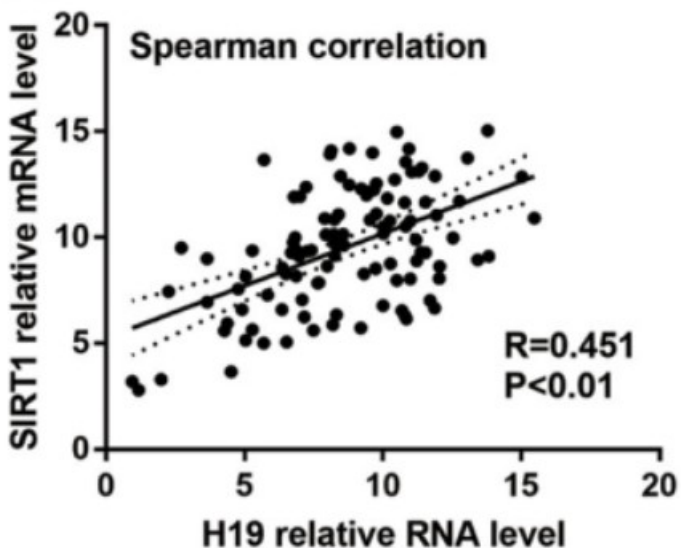
Results of the reversal experiment showed that while **H19 down-regulation led to decreased expression of SIRT1**, simultaneous **miR-194-5p down-regulation was able to reverse the inhibition of SIRT1 expression** in HCT8Fu resistant cell line.

H19 sponges miR-194-5p to modulate SIRT1 expression

H19 correlated with SIRT1 expression in patients

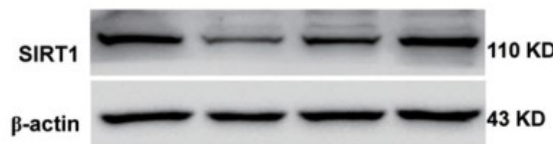


Positive correlation between H19 and SIRT1 expression in CRC tissues.



qRT-PCR

si-NC	+	-	-	+
si-H19	-	+	+	-
miR-194-5p inhibitor	-	-	+	+
NC inhibitor	+	+	-	-



HCT8Fu



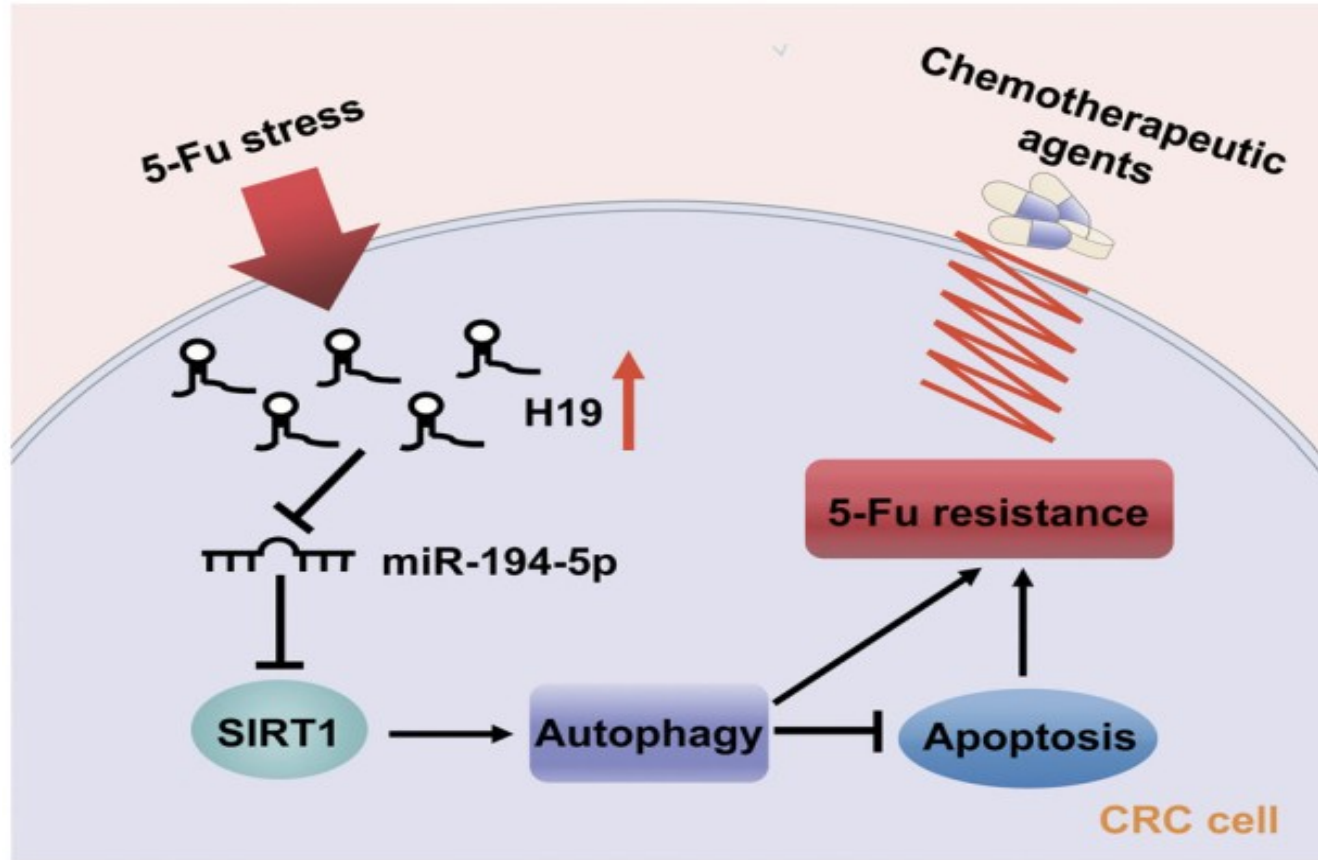
These results confirmed that H19 abolishes the miR-194-5p-mediated repressive activity on SIRT1 by competitively binding miR-194-5p.

pcDNA3.1	+	-	-	+
pcDNA-H19	-	+	+	-
miR-194-5p mimics	-	-	+	+
NC	+	+	-	-



HCT8

H19 sponges miR-194-5p to modulate SIRT1 expression



The functional model underlying the mechanism of H19 on tumor chemoresistance

CONCLUSION

- **H19** is upregulated in CRC recurrence samples appears to be a **potential biomarker** for predicting **5-Fu chemoresistance**.
- **SIRT1 dependent autophagy pathway** could affect **5-Fu chemoresistance in colon cancer cells**, which was modulated by **H19/miR-194-5p axis**.
- As the amount of **H19** is associated with the risk of CRC recurrence, the **measurement of H19 post-surgery may be an effective approach to predict patients' outcome**

Are conventional chemotherapeutic regimens including 5-Fu suitable for CRC patients with a high amount of H19?



CRC patients with a high amount of H19 may be treated with conventional chemotherapy in combination with anti-H19 treatment and/or an autophagy inhibitor.

Bibliography

- Bermúdez, M., Aguilar-Medina, M., Lizárraga-Verdugo, E., Avendaño-Félix, M., Silva-Benítez, E., López-Camarillo, C., & Ramos-Payán, R. **LncRNAs as Regulators of Autophagy and Drug Resistance in Colorectal Cancer.** *Frontiers in oncology.* (2019); 9: 1008.
- Wang M, Han D, Yuan Z, Hu H, Zhao Z, Yang R, et al. . **Long non-coding RNA H19 confers 5-Fu resistance in colorectal cancer by promoting SIRT1-mediated autophagy.** *Cell Death Dis.* (2018) 9:1149.
- Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. **Cancer drug resistance: an evolving paradigm.** *Nat Rev Cancer.* (2013); 13:714–726.
- Nordin M, Bergman D, Halje M, Engstrom W, Ward A. Epigenetic regulation of the Igf2/H19 gene cluster. *Cell Prolif.* (2014); 47: 189–199.
- Chen, Q. N., Wei, C. C., Wang, Z. X., & Sun, M. Long non-coding RNAs in anti-cancer drug resistance. *Oncotarget.* (2017); 8 (1): 1925–1936.
- Cornelison, R., Llana, D. C., & Landen, C. N. Emerging Therapeutics to Overcome Chemoresistance in Epithelial Ovarian Cancer: A Mini-Review. *International journal of molecular sciences.* (2017); 18 (10): 2171.
- Koukourakis, G. V., Kouloulis, V., Koukourakis, M. J., Zacharias, G. A., Zambis, H., & Kouvaris, J. Efficacy of the oral fluorouracil pro-drug capecitabine in cancer treatment: a review. *Molecules (Basel, Switzerland).* (2008); 13 (8): 1897-1922.
- Hibi K, Nakamura H, Hirai A, Fujikake Y, Kasai Y, Akiyama S, et al. Loss of H19 imprinting in esophageal cancer. *Cancer Res.* (1996) 56:480–2.
- Lecerf C, Le Bourhis X, Adriaenssens E. The long non-coding RNA H19: an active player with multiple facets to sustain the hallmarks of cancer. *Cell Mol. Life Sci.* (2019)
- Belharazem D, Kirchner M, Geissler F, Bugert P, Spahn M, Kneitz B, Riedmiller H, Sauer C, Kuffer S, Trojan L, et al. Relaxed imprinting of IGF2 in peripheral blood cells of patients with a history of prostate cancer. *Endocr Connect.* (2012);1:87–94.
- Wu KF, Liang WC, Feng L, Pang JX, Waye MM, Zhang JF, et al. H19 mediates methotrexate resistance in colorectal cancer through activating Wnt/beta-catenin pathway. *Exp Cell Res.* (2017) 350:312–7.
- Liang WC, Fu WM, Wong CW, Wang Y, Wang WM, Hu GX, et al. The lncRNA H19 promotes epithelial to mesenchymal transition by functioning as miRNA sponges in colorectal cancer. *Oncotarget.* (2015) 6:22513–25.



THANKS FOR YOUR ATTENTION