iScience, Volume *26*

Supplemental information

Lupeol synergizes with 5-fluorouracil to combat c-MET/EphA2 mediated chemoresistance in triple negative breast cancer

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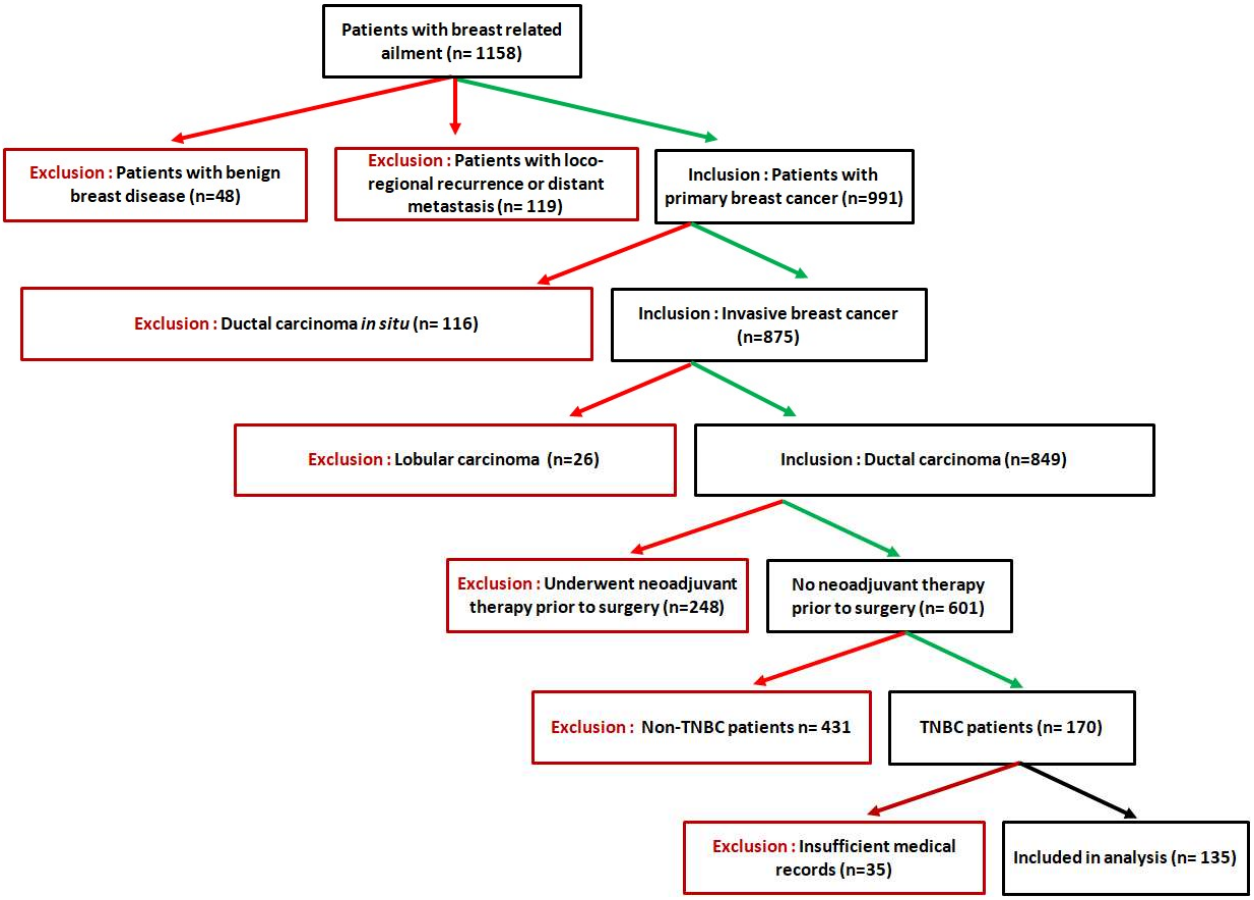
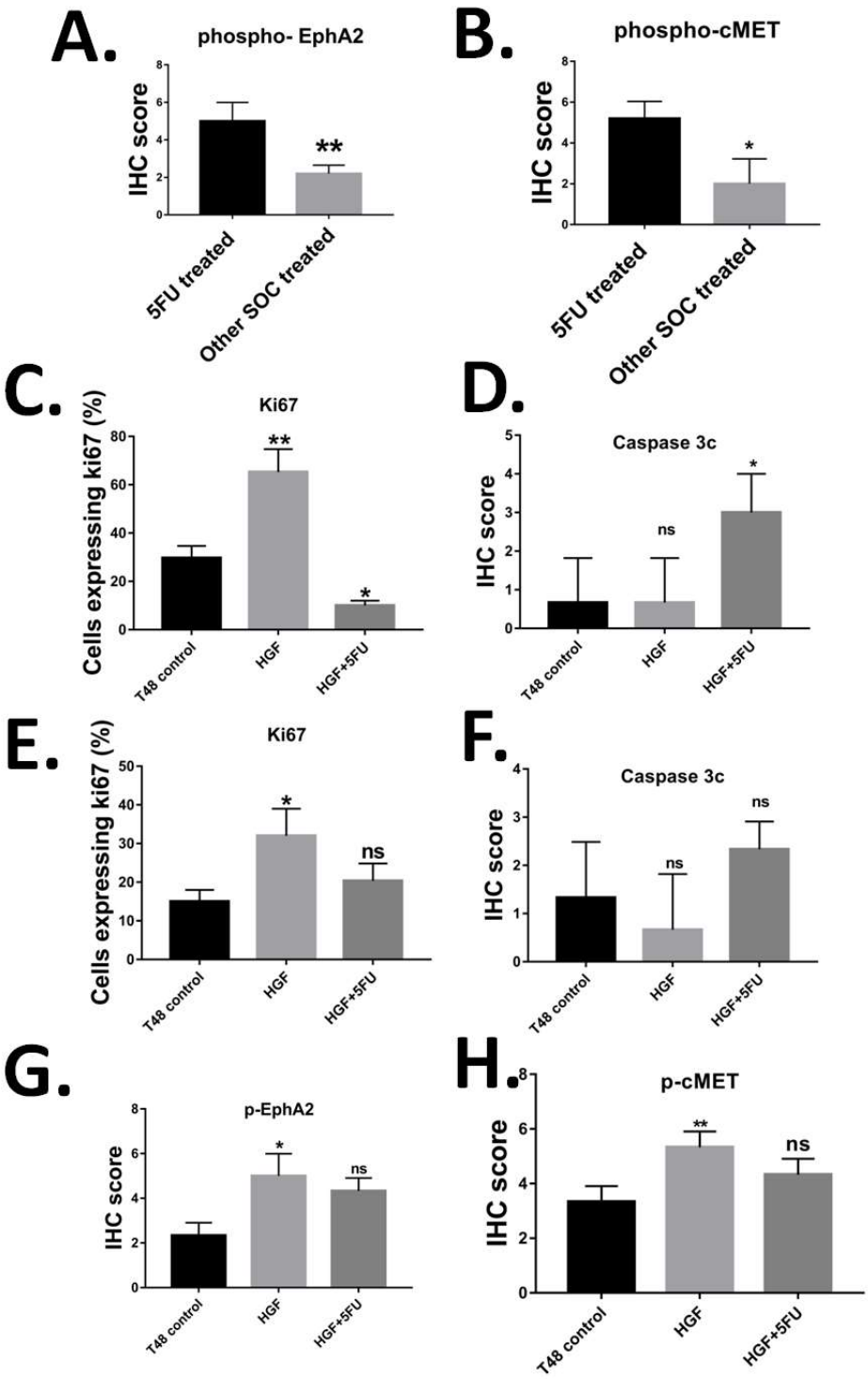
Supplementary Figures

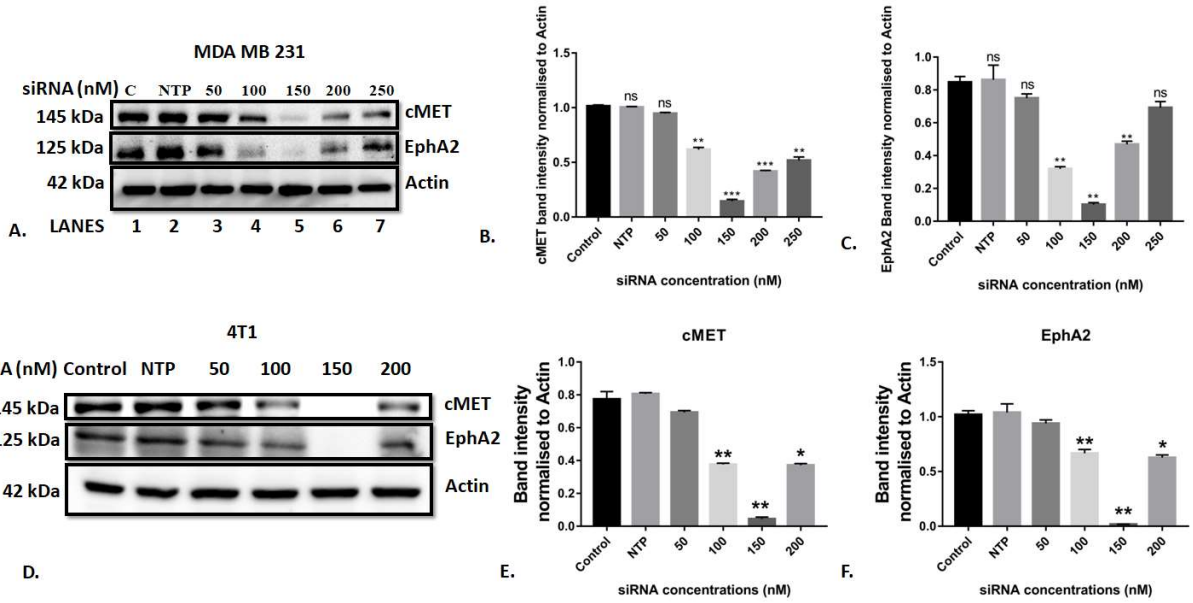


Supplementary Fig. S1: Flow chart describing the inclusion/exclusion criteria of patients in our study. Pertaining to Figure 1.

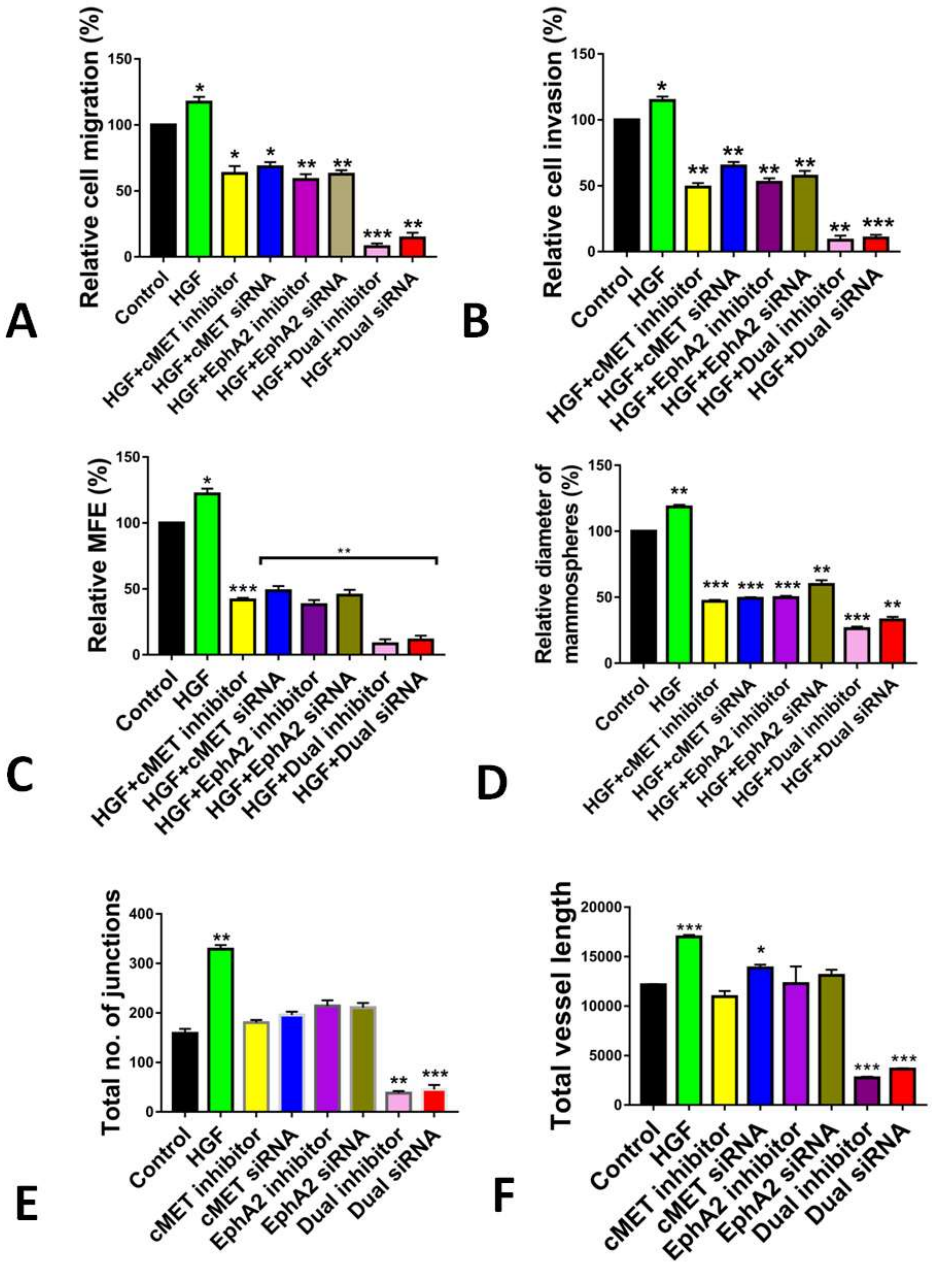


Supplementary Fig. S2: Graphs representing the IHC scores vs various treatment arms. A) and B) Graphs representing the differential IHC score of phospho-EphA2 and phospho-cMET respectively in the 5FU treated and other SOC treated group. C) Ki67 in responders,D) Caspase 3c in responders, E) Ki67 in non-responders, F)

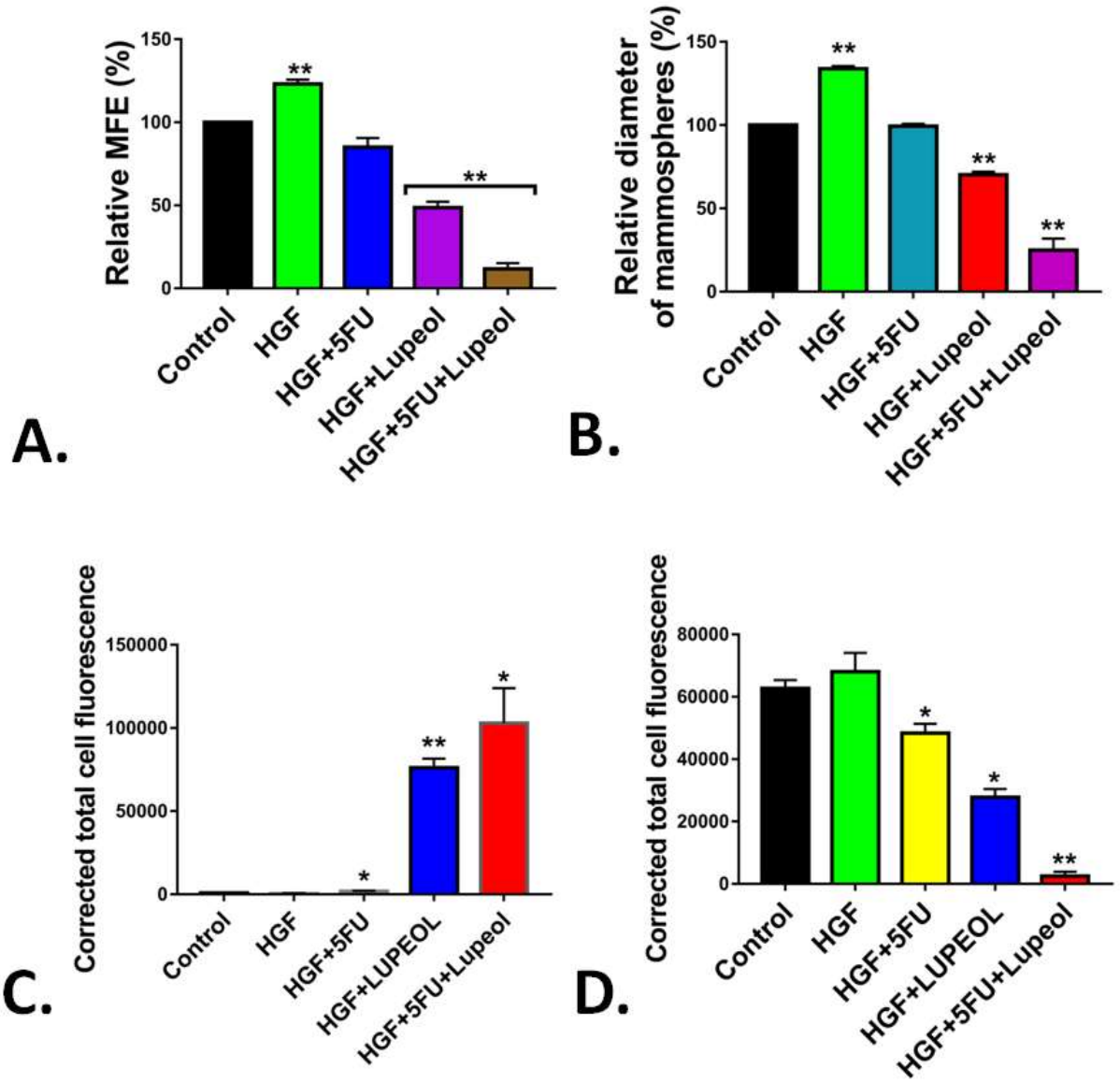
Caspase 3c in non-responders, G) and H) phospho-EphA2 and phospho-cMET in non-responders. *p<0.05, **p<0.02 and ***p<.0.001 statistically significant difference compared to corresponding control. ns= not significant. Related to Figure 1.



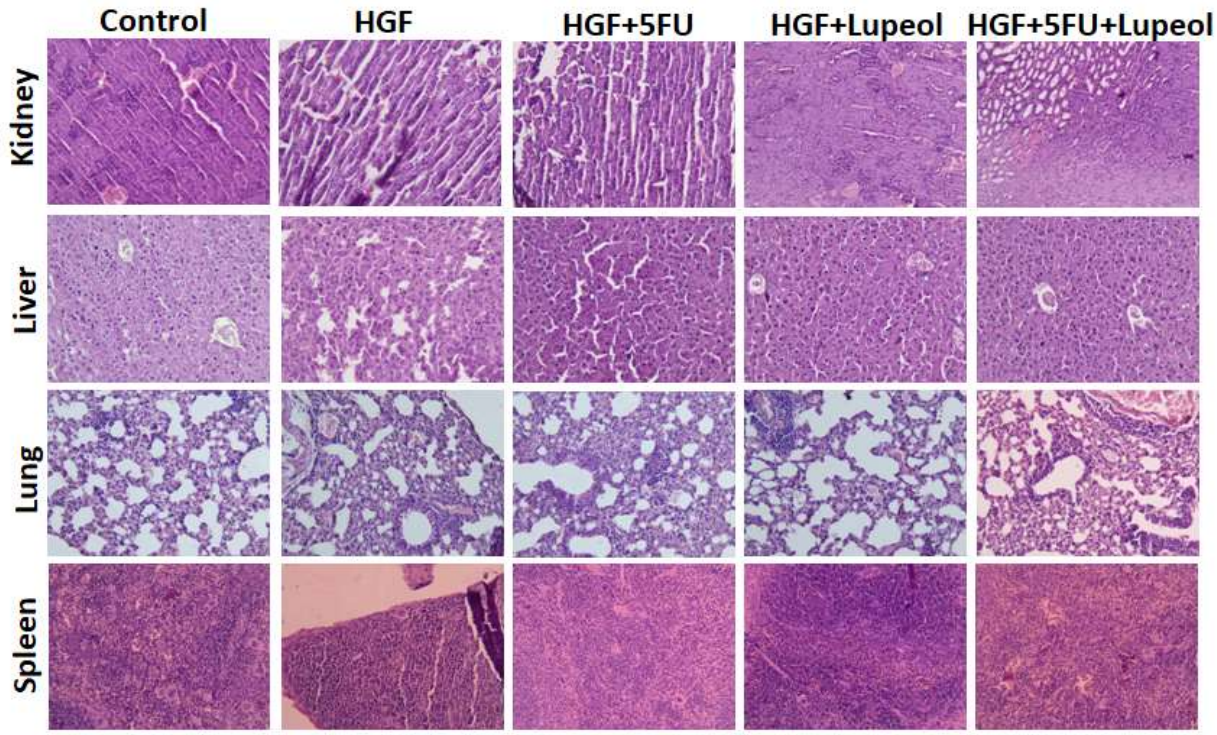
Supplementary Fig. S3: Representative image depicting evaluation of siRNA mediated silencing by Western blot post- transfection. A) Western blot bands of c-MET and EphA2 post- silencing at various concentrations on MDA MB 231 cells. B) and C) Graph depicting normalised band intensity of c-MET and EphA2, respectively, post-transfection with siRNA at various concentrations. Actin was used as loading control. D) Western blot bands of c-MET and EphA2 post- silencing at various concentrations on 4T1 cells. E) and F) Graph depicting normalised band intensity of c-MET and EphA2, respectively, post- transfection with siRNA at various concentrations. Actin was used as loading control. Data are representative of triplicate experiments (mean±SD). *p<0.05, **p<0.02 and ***p<.0.001 statistically significant difference compared to corresponding control by one-way ANOVA. ns= not significant. Pertaining to Figure 2.



Supplementary Fig. S4: Quantitative graphs comparing the effects of siRNA mediated silencing in MDA-MB-231 cell migration, invasion, mammosphere and tube formation. A) and B) Graphs representing the differential relative cell migration and invasion respectively in various groups C) Graph depicting the differential relative mammosphere forming efficiency (%) of MDA-MB-231 cells in various groups. D) Graph depicting the differential relative diameter of mammosphere (%) of MDA MB cells in various groups. E) Graph depicting the differential number of junctions in the tube formation assay. F) Graph depicting the differential number of vessel lengths. Data are representative of triplicate experiments (mean+SD). *p<0.05, **p<0.02 and ***p<.0.001 statistically significant difference compared to corresponding control by one-way ANOVA. Related to Figure 2.



Supplementary Fig. S5: Quantitative graphs comparing the effects of 5FU and/or Lupeol in the presence of HGF on MDA-MB-231 cell's mammosphere forming potential and reversal of EMT. A) Relative mammosphere forming efficiency (MFE) of MDA MB 231 cells in various treatment arms. B) Graphs representing the relative diameter of mammospheres of MDA MB 231 cells. C) and D) Graph depicting the Corrected total cell fluorescence (CTCF) values of E-cadherin and Vimentin expression respectively, upon treating MDA-MB-231 cells with HGF, 5FU, Lupeol or in combination. Data are representative of triplicate experiments (mean±SD). *$p<0.05$, **$p<0.02$ and ***$p<.0.001$ statistically significant difference compared to corresponding control by one-way ANOVA. Pertaining to Figure 5.



Supplementary Fig. S6: Histopathological evaluation of the effect of the various treatment arms by haematoxylene and Eosin staining of Kidney, liver, lung and Spleen of mice after they were treated with HGF, 5FU and Lupeol, alone or in combination. Pertaining to Figure 6.

Supplementary tables

Parameters	N (%)
Age at diagnosis (years)	
≤ 50	64 (47.41)
>50	71 (52.59)
T stage	
T1	41 (30.37)
T2	35 (25.92)
T3	25 (18.52)
T4	34 (25.19)
N stage	
N0	64 (47.41)
N1	45 (33.33)
N2	16 (111.85)
N3	10 (7.41)

Supplementary table S1: Demographic and pathological profiles of TNBC patients (N=135). Pertaining to Figure 1.

Parameters	N (%)
Age at diagnosis (years)	
≤ 50	6 (40)
>50	9 (60)
T stage	
T1	3 (20)
T2	7 (46.67)
T3	2 (13.33)
T4	3 (20)
N stage	
N0	8 (53.34)
N1	5 (33.33)
N2	2 (13.33)
N3	0 (0)

Supplementary table S2: Demographic and pathological profiles of TNBC patients (N=15) used for ex vivo explant culture. Pertaining to Figure 1.

	pEphA2 positive	pEphA2 negative	P value
5FU treated [N (%)]	24 (75)	8 (25)	
Other SOC [N (%)]	3 (23.08)	10 (76.92)	0.001271*

Supplementary table S3: Association between the differential expressional status of phospho-EphA2 in the 5FU/Other SOC treated cohort. *p<0.05 was considered to be statistically significant by chi-square test. Pertaining to Figure 1.

	p-cMET positive	p-cMET negative	P value
5FU treated [N (%)]	21 (75)	11 (25)	
Other SOC [N (%)]	4 (23.08)	9 (76.92)	0.032944*

Supplementary table S4: Association between the differential expressional status of phospho-cMET in the 5FU/Other SOC treated cohort. *p<0.05 was considered to be statistically significant by chi-square test. Pertaining to Figure 1.

	Non- responder	responder	P value
pEphA2 levels not changed [N (%)]	7 (75)	1 (25)	0.020*
pEphA2 levels decreased [N (%)]	2 (23.08)	5(76.92)	

Supplementary table S5: Association between the differential expressional status of phospho-EphA2 levels in the responder and non-responder groups. *$p<0.05$ was considered to be statistically significant by chi-square test. Pertaining to Figure 1.

	Non- responder	responder	P value
pMET levels not changed [N (%)]	8 (80)	2 (20)	0.025*
pMET levels decreased [N (%)]	1 (25)	4(75)	

Supplementary table S6: Association between the differential expressional status of phospho-cMET levels in the responder and non-responder groups. *$p<0.05$ was considered to be statistically significant by chi-square test. Pertaining to Figure 1.

	MDA-MB 231
Factors	**Lupeol+ 5FU**
Dose selection (µM)	**Lupeol**: 2,4,8,10,15,20 **5FU**:5, 10, 15
IC 50 for individual compound treatment (µM)	**Lupeol**: 29.54 5FU: 37.13
IC 50 for combined treatment (µM) (Effective Dose)	**Lupeol**: 8.651 when **5FU** 10
Combination Index (CI) **CI = ($D_{Lupeol, Comb}$) / (D_{Lupeol}) + ($D_{5FU, Comb}$) / (D_{5FU})**	**C1**= (8.651/29.54)+ (10/37.13) = 0.562
Dose reduction Index (DRI) **D_{Drug} / $D_{Drug, Comb}$**	**Lupeol**: (29.54/8.651)= 3.41 **5FU**= (37.13/10)= 3.71
Combination effect	**Synergistic**

Supplementary table S7: Determination of effective dose and combination effect. IC50 = 50% cell growth inhibition after treatment with certain drug. $D_{Lupeol\ (\mu M)}$ = Dose of Lupeol to affect cell growth inhibition when treated individually. $D_{5FU,\ Comb\ (\mu M)}$= Dose of 5FU to affect cell growth inhibition when treated in combination with Lupeol. $D_{Lupeol,\ Comb\ (\mu M)}$= Dose of Lupeol to affect cell growth inhibition when treated in combination with 5FU. $D_{5FU\ (\mu M)}$= Dose of 5FU to affect cell growth inhibition when treated individually. The combination index (CI) determined by using Chou–Talalay method. The CI values <0.9: synergistic; values 0.9–1.1: additive, and values >1.1: antagonistic were considered for the effect of combinations of two compounds as described by Chou (2010). The dose-reduction index (DRI) is a measure of dose reduction of each drug in a synergistic combination at a given effect level, compared with the doses of each drug alone calculated according to using Chou–Talalay method. Related to Figure 3.

Parameters	Untreated Control	Vehicle Control	HGF	HF	HL	HFL
UR(mg/dL)	37.84±3.42	38.12±4.25	38.72±5.28	39.55±3.56	37.52±4.23	38.29±2.15
UA (mg/dL)	3.01± 0.18	3.25±0.12	3.16±0.22	3.56±0.45	3.23±0.18	3.38±0.19
GLB (g/dL)	3.82±1.62	3.75±0.81	3.77±1.26	3.96±1.36	3.80±1.02	3.78±1.19
TP (g/dL)	5.45±1.85	5.51±1.95	5.59±2.05	5.32±2.20	5.41±0.98	5.61±1.58
ALB (g/dL)	1.82±0.55	1.85±0.61	1.90±0.58	1.95±0.91	1.86±0.68	1.88±0.69
CHL (mg/dL)	80.18±30.29	81.28±26.38	82.15±32.58	78.29±45.41	81.45±32.84	79.12±20.58
TG (mg/dL)	72.58±28.25	71.26±25.58	72.42±21.98	71.59±36.15	73.61±29.54	71.22±26.24
GLC (mg/dL)	152±21.97	150.24±18.65	151.29±26.57	154.48±20.35	153.68±20.02	156.41±23.68
AP (UI/L)	384±48.21	386±56.21	381±42.38	376±56.28	385±35.85	383±38.69
AST (UI/L)	162±33.50	162±31.29	164±26.21	169±30.18	159.25±18.68	160±25.98
ALT (UI/L)	245.96±38.94	250.31±40.18	257±32.04	262.91±39.12	249.21±35.06	256.28±42.12

Supplementary table S8: Various serum parameters of mice after treatment with HGF, 5FU or Lupeol, alone or in combination. Abbreviations: ALB= albumin; ALT= alanine transaminase; AP= Alkaline phosphatase; AST= aspartate transaminase; CHL= cholesterol; GLB= globulin; GLC= glucose; TG= triglycerides; TP= total protein; UA= uric acid; UR= urea. Data are representative of triplicate experiments (mean±SD). Related to Figure 6.

Parameters	N (%)
Age at diagnosis (years)	
≤ 50	3 (42.86)
>50	4 (57.14)
T stage	
T1	1(14.29)
T2	2 (28.57)
T3	2 (28.57)
T4	2 (28.57)
N stage	
N0	3 (42.86)
N1	2 (28.57)
N2	2 (28.57)
N3	0 (0)

Supplementary table S9: Demographic and pathological profiles of TNBC patients (N=7) used for ex vivo explant culture related to main Figure 7 H.