CARACTERIZAREA MOLECULARA A SEGMENTELOR GENOMICE IMPLICATE IN TRANSFORMAREA LA NIVELUL CELULEI STEM HEMATOPOIETICE IN NEOPLASMELE MIELOPROLIFERATIVE BCR-ABL NEGATIVE.

DE LA DETECTIE LA TERAPIE SELECTIVA

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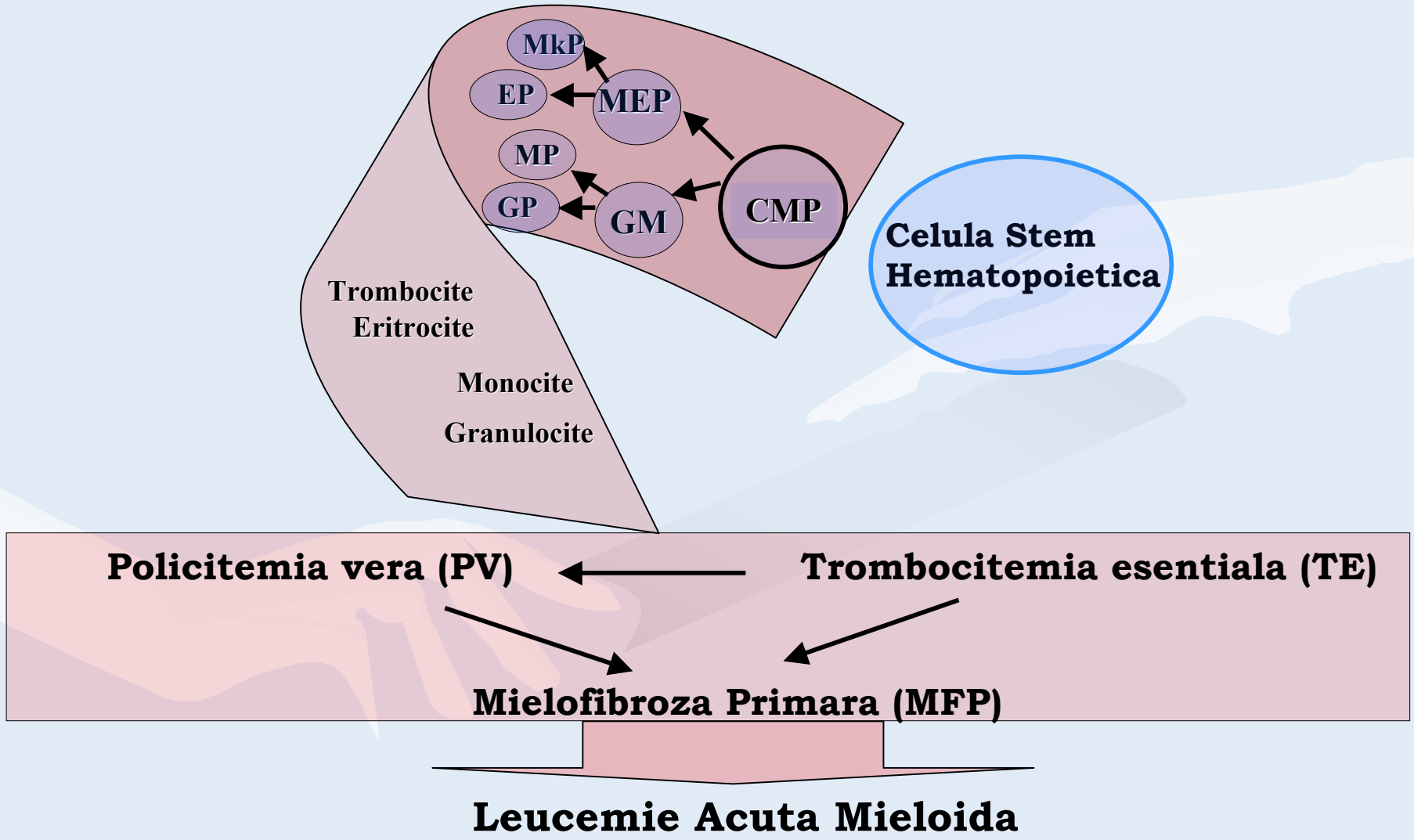
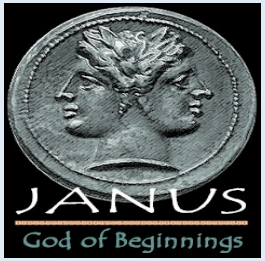
Neoplasmele Mieloproliferative BCR-ABL Negative

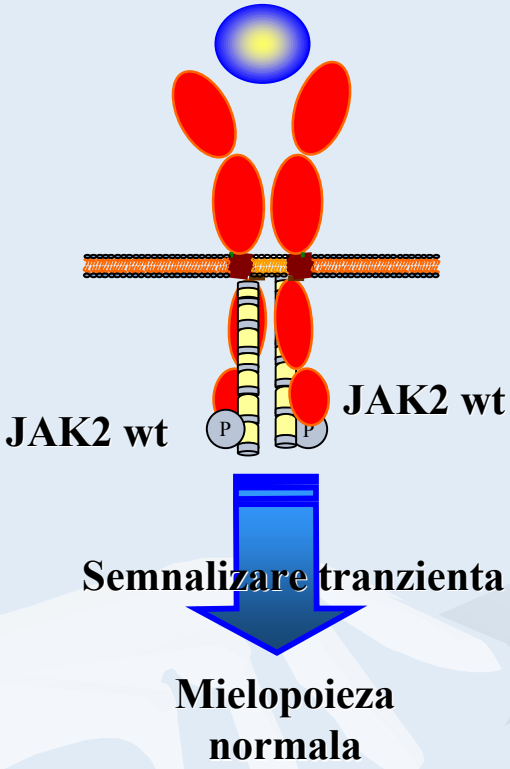


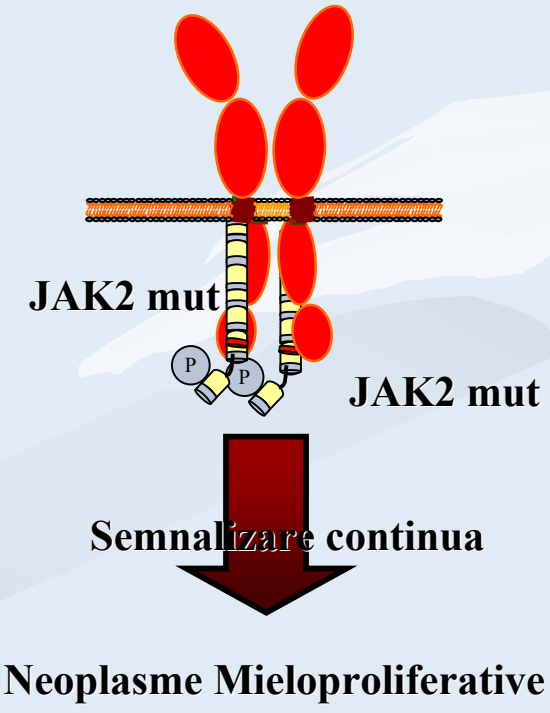
Ipoteza stiintifica si semnificatie



Kinazele Janus(JAK) joaca un rol esential in semnalizarea prin intermediul membrilor superfamiliei receptorilor pentru citokine

- Proliferation
- Differentiation
- Transformation





***JAK2* V617F este evenimentul molecular major in vasta majoritate a pacientilor cu PV si >50% a pacientilor cu TE sau MFP**

Design si Metode

Diagnostic de precizie

Studii clinice aprofundate

- Elucidarea mecanismelor fiziopatologice
- **Treatmente eficiente**

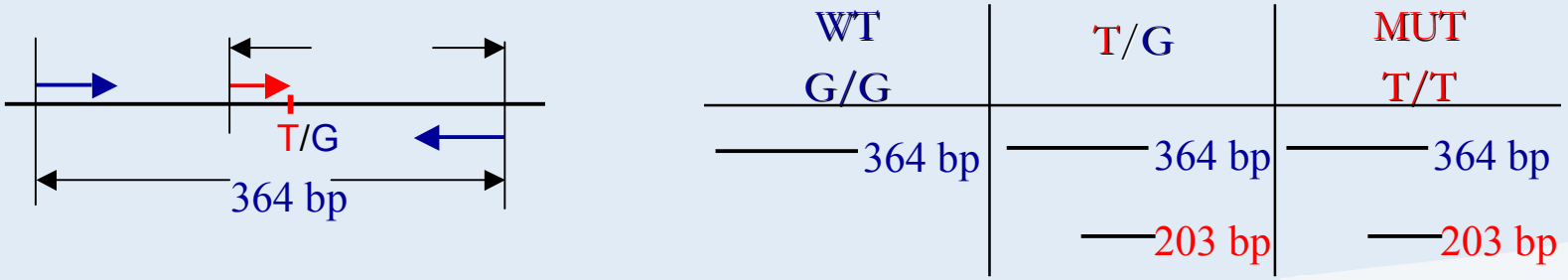
Patients

254 patienti diagnosticati cu NMP BCR/ABL negative la Spitalele Clinice **Coltea si Fundeni** au fost investigati pentru prezenta mutatiilor in genele **JAK2** si **TpoR (Mpl).**

	No. of patients	F	M	Median age (range)
PV	88	32	56	57 (17 - 87)
ET	137	88	49	51 (7 - 87)
PMF	29	12	17	54 (33 - 81)

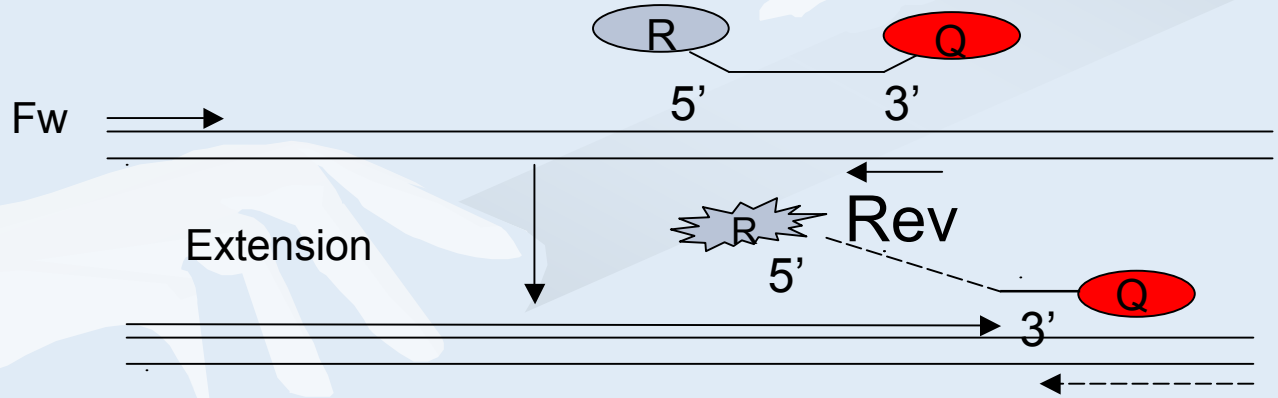
- **PCR cu specificificitate alelica AS- PCR**

-utilizeaza un primer specific pentru alela mutanta si primeri pentru controlul intern



- **TaqMan pentru discriminare alelica**

- utilizeaza o sonda ce se bazeaza pe activitatea 5'-3' nucleazica a polimerazei ADN Taq si primeri specifici pentru fiecare alela

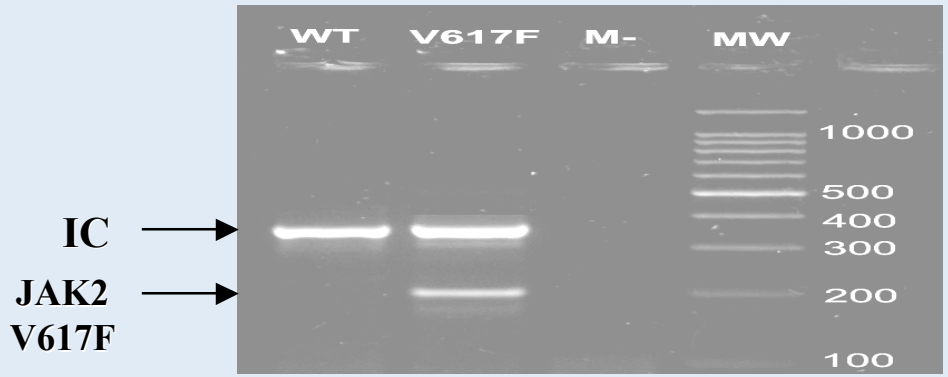


- **Secventiere ADN**

-utilizeaza un primeri specifici pentru amplificarea exonilor de interes (12, 14 in JAK2; 10 in MPL) si tehnologia BigDye Terminator v3.1.

REZULTATE

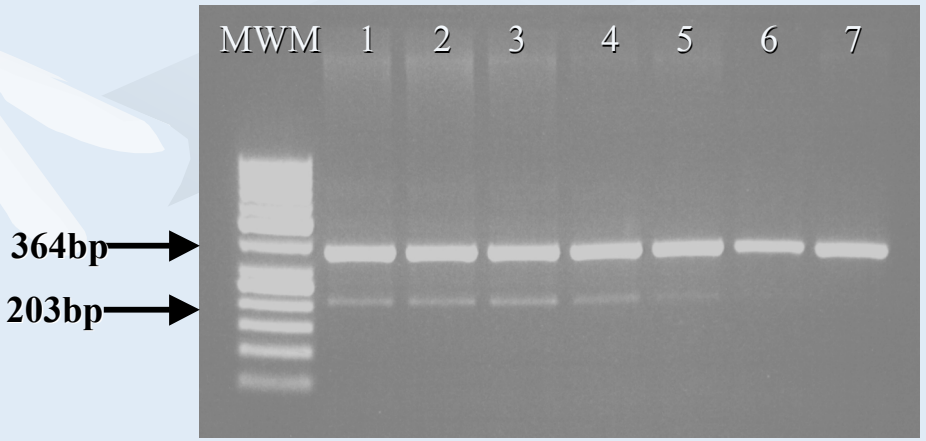
PCR cu specificitate alelica (AS- PCR)



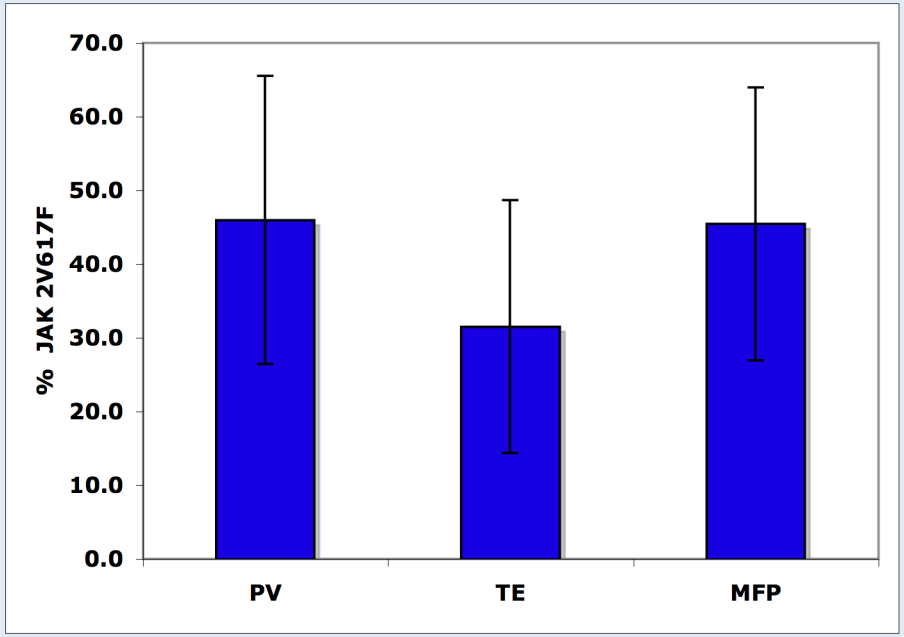
JAK2 WT – G/G
JAK2 V617F – T/G
M- - control negativ de amplificare
MW - marker de greutate moleculara

Sensibilitatea AS- PCR < 1%

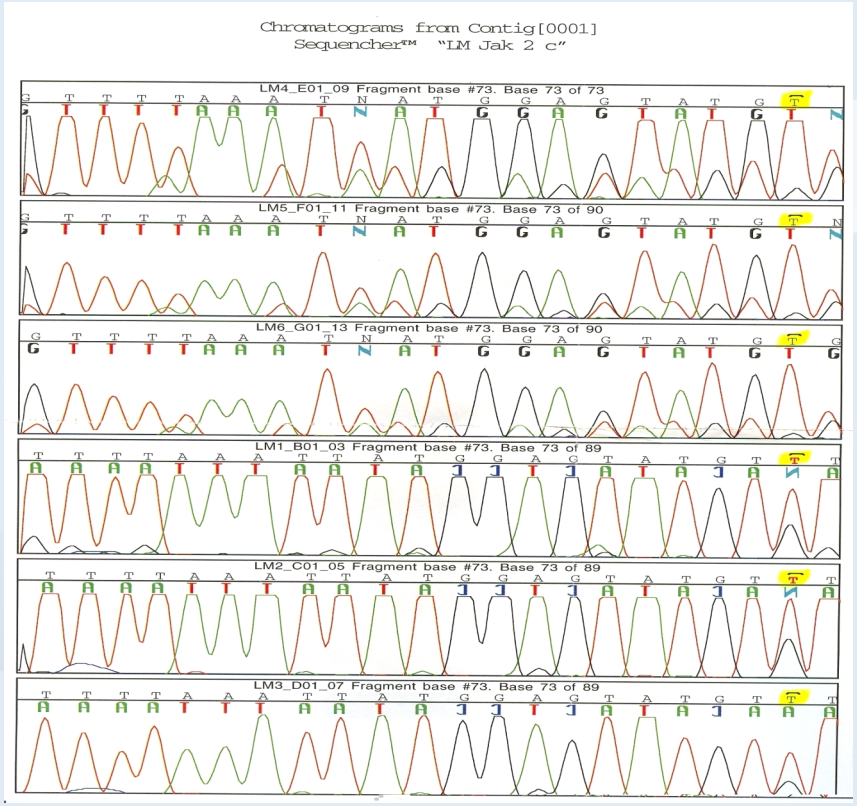
1. JAK2 V617F 100%
2. JAK2 V617F 50%
3. JAK2 V617F 10%
4. JAK2 V617F 5%
5. JAK2 V617F 1%
6. JAK2 V617F 0.1%
7. JAK2 WT 100%



% JAK2 V617F la pacienti cu NMP (TaqMan)

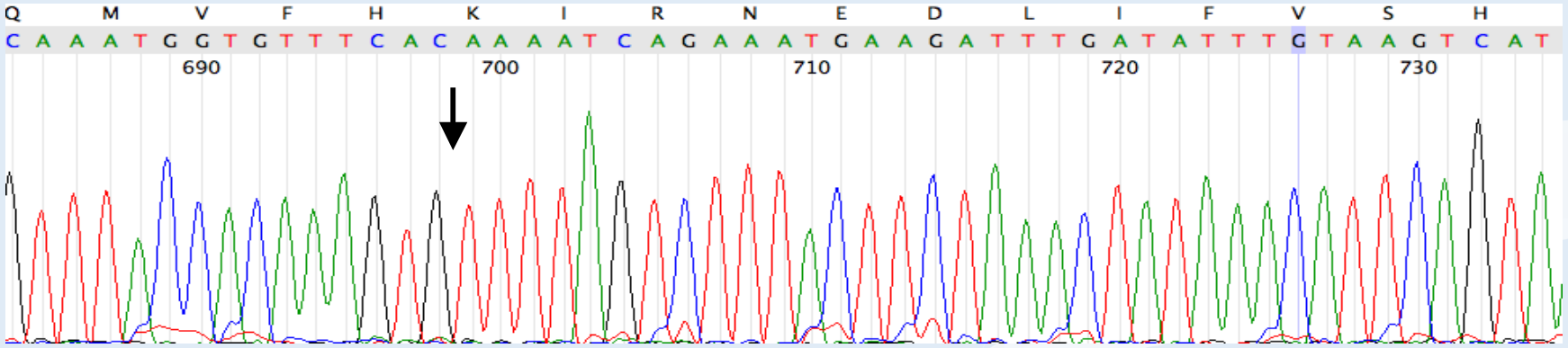


Secventierea JAK2 Exon 14



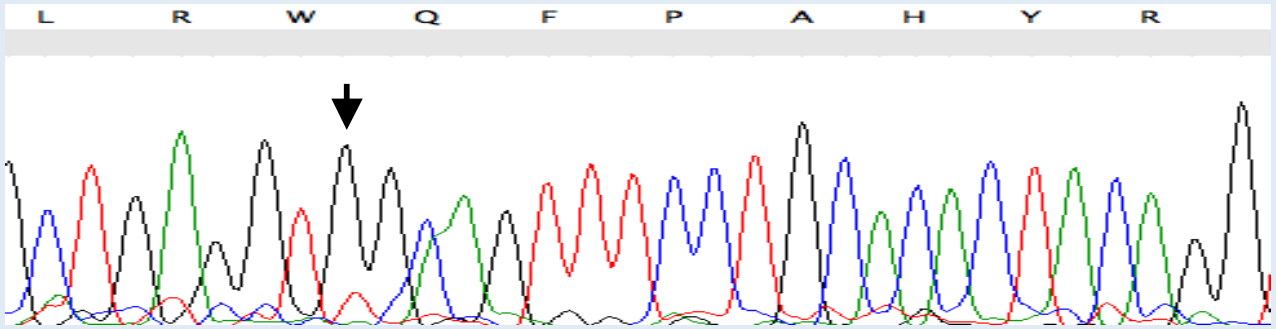
Secvente aliniate pentru un pacient heterozigot JAK2 V617F (T/G)
(trei replicate pentru fiecare primer).

Secventierea JAK2 Exon 12

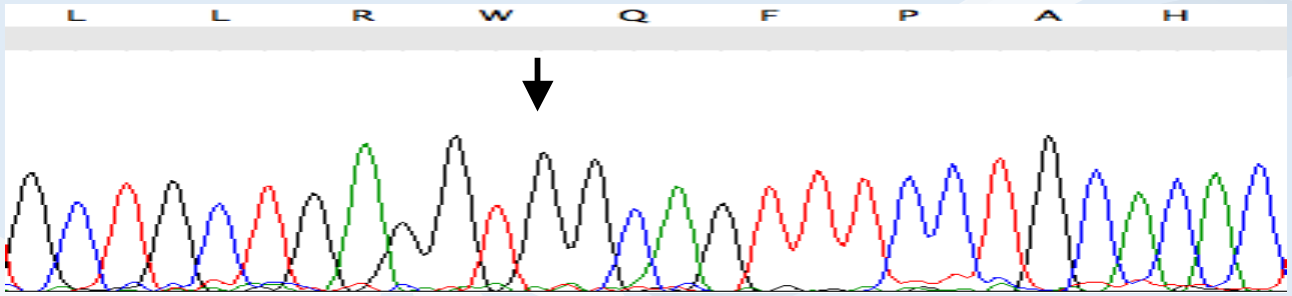


Secventierea **JAK2 Exon 12** pentru un **pacient cu PV** homozigot pentru alela wt **K539**.

Secventierea MPL Exon 10



Secventierea **Exonului 10 TpoR (MPL)** pentru un pacient cu MFP heterozigot pentru mutatia **W515L** (TGG > TTG).



Secventierea **Exonului 10 TpoR (MPL)** pentru un pacient cu MFP homozigot pentru alela wt **W515** (TGG).

Concluzii I

- **Mutatia JAK2 V617F este prezenta la majoritatea pacientilor**

	No. of patients	F	M	Median age (range)	V617F positive no. (%)	WT	other JAK2 mutations	MPL W515L
PV	88	32	56	57 (17 - 87)	69 (78.4%)	19	2	0
ET	137	88	49	51 (7 - 87)	68 (49.6%)	69	0	0
PMF	29	12	17	54 (33 - 81)	18 (62%)	11	1	1

- **Alte mutatii cunoscute in JAK2 si MPL, au frecventa redusa in lotul nostru, dar se poate presupune ca mutatii noi vor fi identificate.**

- **PV si MFP au % al alelei JAK2V617F semnificativ mai mare decat TE ($p<0.05$).**

Diagnostic de precizie

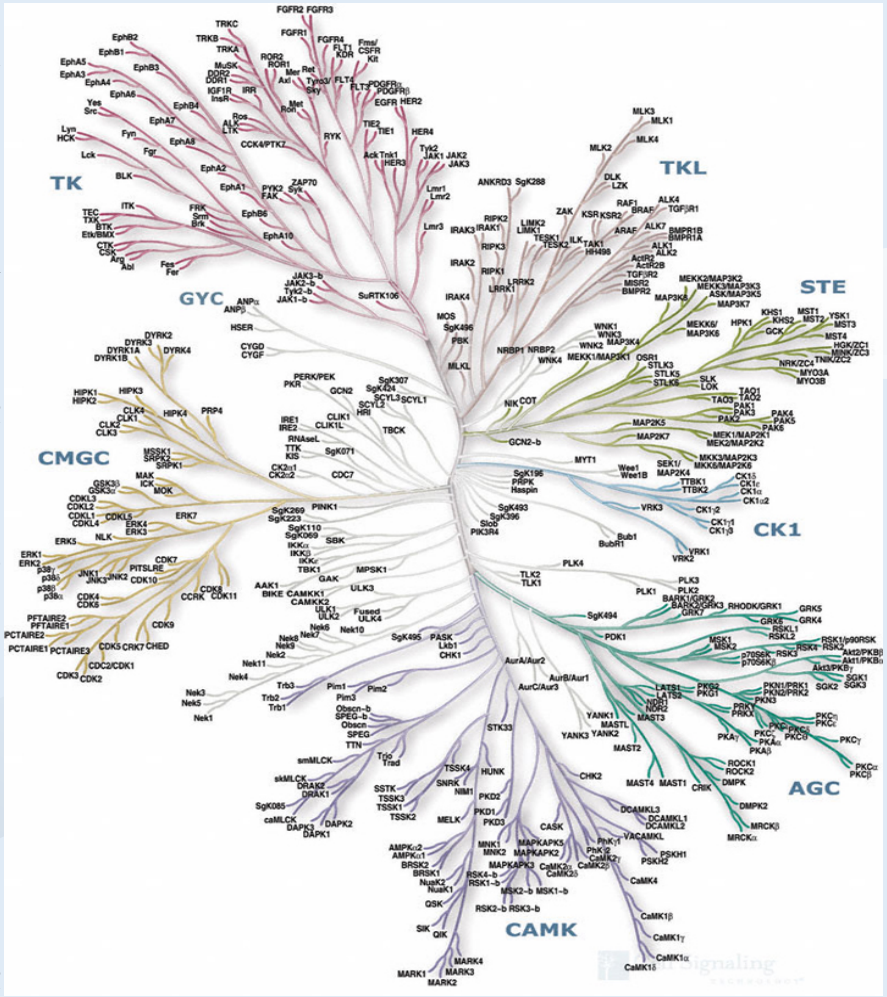
Studii clinice aprofundate

- **Elucidarea mecanismelor fiziopatologice**

- **Treatmente eficiente**

De ce inhibitorii kinazici sunt intens investigati in industria farmaceutica?

- **Aproximativ 2% din genomul uman codifica protein kinaze (PK) = Kinom;**
- **Complexitatea kinomului ofera numeroase oportunitati pentru dezvoltarea unor noi inhibitori**
- **478 kinaze au domenii catalitice inalt conservate;**
- **PK joaca roluri cheie in reglarea mecanismelor moleculare;**
- **PK catalizeaza fosforilarea reversibila a > 10,000 proteine la > 500,000 situri;**
- **PK sunt implicate in mai mult de 400 maladii;**
- **In ultimii 20 ani de cercetare oncologica au fost identificate aprox. 100 oncogene, cele mai multe dintre ele fiind tyrosine kinaze (TK).**



Kinomul Uman.

Reprodus cu acordul Cell Signaling Technology

Sistemele celulare reprezinta o modalitate atractiva de caracterizare a genelor si/sau proteinelor tinta si a activitatii unor compusi

Un panel de sisteme celulare dependente sau independente de kinaze

↓

Colectii moleculare (small-molecule compounds)

↓

Screeningul inhibitiei proliferarii

↓

Candidati

↓

Confirmarea activitatii
Teste de apoptoza/necroza
Teste cu reporteri genetici

↓

Investigarea mecanismului de actiune

Testare clinica

Ba/F3

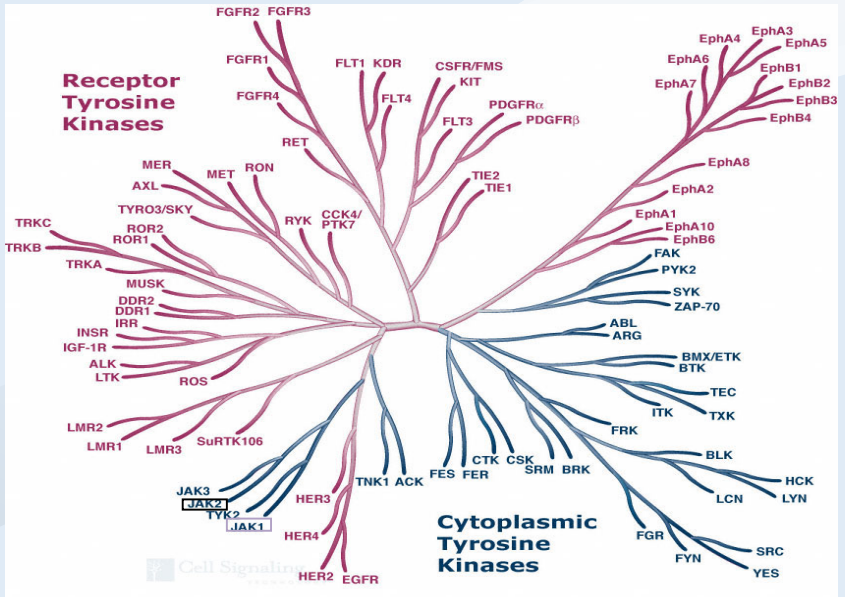
Ba/F3 JAK2 wt

→ **dependente de IL-3 pentru proliferare si supravietuire**

Ba/F3 JAK2 V617F

Ba/F3 JAK1 V658F

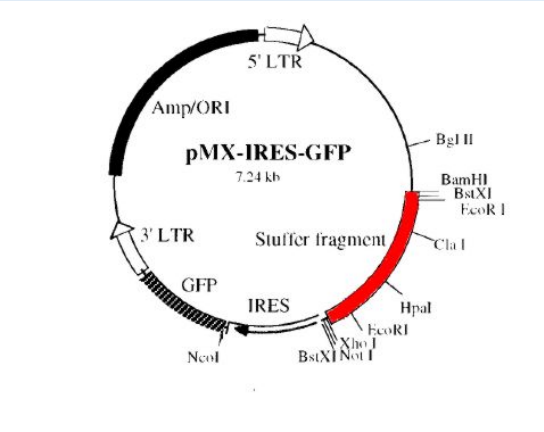
→ **Independente de IL-3 datorita TK mutate**



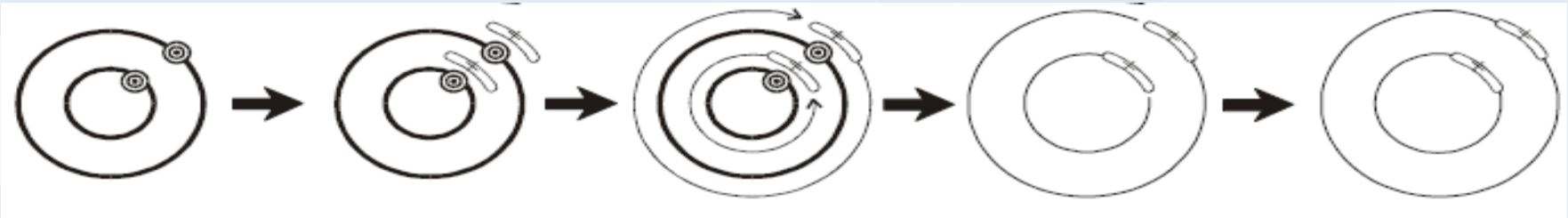
Tirozinkinomul.
Tirozin kinazele utilizate in testele de screening sunt marcate pe diagrama filogenetica reprezentand cele 90 tirozin kinaze din tirozinkinom.

Generarea panelului de linii celulare hematopoietice necesar screeningului

Vectorul Retroviral Bicistronic pMX-IRES-GFP



ADNc corespunzatoare genelor mutante **JAK2V617F** si **JAK1 V658F** au fost obtinute prin **mutageneza in-situ (Quick Change Site-directed Mutagenesis)**.



1.Introducerea ADNc JAKwt in pMX-IRES-GFP

2. Denaturare si asociere cu primerii continand mutatia

3. Elongarea primerilor in prezenta polimerazei *PfuTurbo*

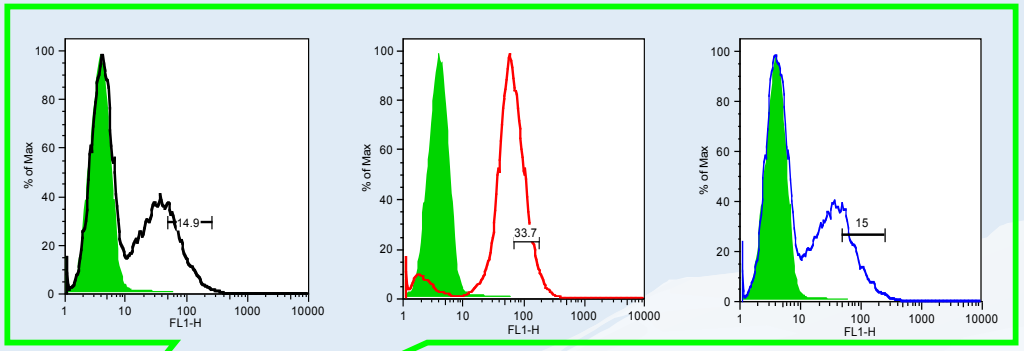
4. Digestia plasmidei parentale metilate utilizand D*pnI*

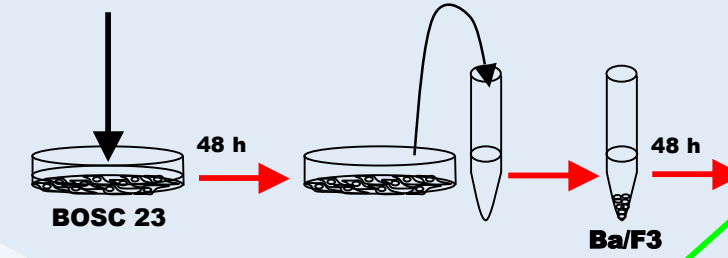
5. Transformarea ADNds in Celule bac supercompetente

Verificarea ADNc s-a realizat prin secventiere

Generarea si validarea panelului de linii celulare utilizate in testele de citotoxicitate

Celulele Ba/F3 infectate retroviral, care exprima JAK2 wt, JAK2 V617F sau JAK1 V658F au fost sortate pentru GFP (>15%) utilizand BD FACSVantage™ SE Cell Sorter.

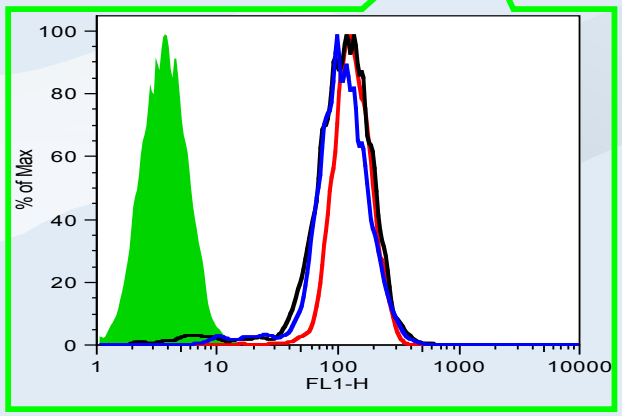




Sortare

Dependenta de IL-3 (Ba/F3 JAK2wt)

Independenta de IL_3 (Ba/F3 JAK2 V617F and Ba/F3 JAK1 V658F)



Nivelul expresiei JAK2wt, JAK2V617F si JAK1V658F este proportional cu cel al GFP

	Mean FL-1H
Ba-F3 Jak2wt GFP	123
BaF3 Jak2V617F GFP	137
Ba-F3 Jak1VtF GFP	120
BaF3	4

Concluzii II

- **Strategia noastra de screening este capabila sa interogheze activitatea unor colectii de compusi asupra unui panel de sisteme celulare, in replicate, in format dependent de doza si poate identifica inhibitori selectivi pentru JAK2 mutant *vs* JAK2wt, tinand cont de inalta omologie si plasticitate structurala a acestor proteine.**

- **Simultan, aceasta strategie genereaza informatii despre potentialii inhibitori pan-JAK sau pan-JAK2.**

- **Screeningul pe sisteme celulare permite o evaluare *in vitro* mentinand conformatia fiziologica a proteinelor tinta si in acelasi timp permitand evaluarea toxicitatii celulare neselective.**

Perspective:

- **Aceasta strategie ar putea fi folosita pentru oricare alta mutatie semnificativa ce ar putea fi identificata si s-ar putea adresa diferitor familii de gene (kinase, protease, receptori, co-receptori, etc.).**

- **Dezvoltarea unor metode HTS pentru analiza relatiei structura-activitate (SAR)**

Echipa de lucru

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