Tumour heterogeneity and tumour progression in NENs
Outlines

• Mention principles of Tumour Heterogeneity, definitions and main practical consequences

• Discuss Intra-Tumour Heterogeneity of NET, from morphology and function, to proliferation and genetics
Tumour Heterogeneity (TH)

• Intra-Tumour Heterogeneity (Intra-TH)

• Inter-Tumour Heterogeneity (Inter-TH)
  • Differences between primary/met or met/met
  • Spatial/synchronous H or temporal/metachronous H

• Inter-Patient Heterogeneity
  – Differences between tumours of the same entity among patients
  • This type of H is evaluated in most studies

TH, in the same patient = Intra-Patient H
Different types of Intra-Patient Heterogeneity

a) Spatial heterogeneity within the primary T
b) H between primary and met
c) Inter-metastatic H
d) Temporal H between met

By simplification, intra-Patient H is called Intra-Tumour H (ITH), because it corresponds to the progression of the same initial tumour
Intra-Tumour Heterogeneity (IntraTH)

- Phenomenon distinct into 2 parts
  - **Clonal H**
    - due to the different distribution of molecular alterations (mutation, methylation, CN alterations)
  - **Non-clonal H**
    - depending on interactions with the microenvironment, reflecting morphological features recognizable in routine H&E (vessels, stroma, inflammatory cells...)

- **Influencing**
  - Morphology and differentiation
  - Immunophenotype
  - Proliferation
  - ...and biological aggressiveness, resistance to therapy

Stanta G et al, Virchows Arch, 2016: 469
CLONAL HETEROGENEITY:

- A genetic event (mutation/copy number alteration) has given a growth advantage
- Heterogeneous subclones emerge, leading to both spatial and temporal H
- Subclones, subject to selection pressure, can outgrow the remaining tumour:
  - metabolic (hypoxia)
  - environment (vessels, immune..)
  - treatment...

To study minor subclones, it is important to perform spatial and longitudinal sampling.
Subclones resistant to / or modified by targeted drugs can lead to tumour recurrence and progression.

TREATMENT-INDUCED CLONAL H

Genetic events

Heterogeneous subclones

Treatment targeted on some subclones

Tumour recurrence by resistant subclone
Clonal H

Non-clonal H: modifications secondary to interactions with microenvironment

Schematic representation of H in tumour progression: 2 types of H are involved in T progression. Both influence morphology, phenotype, aggressivity of T cells

Adapted from:
Practical Issues of Tumor Heterogeneity
Intra-Tumour Heterogeneity (ITH) practical consequences

• A complex phenomenon, central in tumour development
• Has 3 main key impacts:
  - Basis of acquired drug resistance
  - Major source of low level of reproducibility in clinical cancer research
  - Limits the precision of histological diagnosis, reduces the value of biopsy
ITH and NEN

- What do we know about NEN heterogeneity?
- Most results have described Inter-patient H, few data on intra-tumour H

- Intra-tumour Heterogeneity in NET
  - Important in practice for NET patient care? YES!
    - long-term disease, several metastases, progression, question of new/sequential sampling...
    - several sequential treatments with possible resistance that must be better understood...
    - new potential therapies that could be targeted on different subclones...
Intra-TH in NET different levels

• Morphology
• Function-phenotype
• Molecular alterations (including proliferation)
Intra-TH in NET
different levels

• Morphology
• Function-phenotype

Discuss 4 possible types of ITH in NET
- Morphological (H&E)
- Microvessels (IHC)
- Chromogranin (IHC)
- Hormone secretion (IHC/clinical)
Morphological ITH

Macroscopic and microscopic level (H&E)

Examination of resected specimens show that NET are « classically » homogeneous as compared to other tumours
More frequently homogeneous

Macroscopic level

heterogeneous
Microscopic level (H&E) homogeneous at low or high magnification

Examples of Panc and ileal NETs
In some NET areas of fibrotic stroma, cystic or haemorrhagic modifications. This ITH is important for pathologists for diagnostic in case of biopsy samples.
Microvessel ITH

- Important inter-patient H, with a wide range of values according to prognosis
- Benign NET are highly vascularized
  - Transformation of vessels with progression??
- Important for antiangiogenic drugs

![Microvessel density image]

Marion-Audibert et al, Gastroenterology 2003; 125
Couvelard et al, Br J Cancer 2005;17:94
Core samples selected randomly from paraffin blocks of resected Met
We found substantial variability, indicating high H of intra-met and inter-met vessel density, that increased with time.

Modification of vascularisation with progression in a patient?

Could be assessed by sequential functional imaging?

**Important H of vessel density**

<table>
<thead>
<tr>
<th>Method</th>
<th>ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-Met H</td>
<td>0.48</td>
</tr>
<tr>
<td>Inter-Met H synchronous</td>
<td>0.48</td>
</tr>
<tr>
<td>Inter-Met H metachronous</td>
<td>0.00</td>
</tr>
</tbody>
</table>

ICC: intraclass correlation coefficient; 0 totally unreproducible to 1 perfectly reproducible
Chromogranin A ITH

- Chromogranin A is frequently heterogeneous inside NET
- Frequent in some locations such as pancreas, rare in ileum
- H at the cellular level, or within areas
Case: CGA heterogeneity in a separate area in a 9 cm primary pancreatic NET

Synaptophysin homogeneous

ChromograninA

Images: Dr Jérôme CROS
Unknown role of absence or presence of CGA
We found different morphology/architecture in CGA+ and CGA- areas, on HE

Images: Dr Jérôme CROS
And also different proliferation areas: link with aggressiveness?

\[ \text{Ki-67} = 2\% \]

\[ \text{CGA+ area} \]

\[ \text{Ki-67} = 30\% \]

\[ \text{CGA} - \text{area} \]

Images: Dr Jérôme CROS
Hormonal profile can change over time
- N=435 pancreatic NET
- 3.4% metachronous functional syndrome (m=55 mths, 7-219)
- Insulin (n=5), VIP (n=5), gastrin (n=2), glucagon (n=4)

MHS mainly occurred in the setting of increased tumour aggressiveness, progression, proliferation
Summary morpho-functional H in NET

• A relatively low degree of spatial and temporal heterogeneity in NET
  – This type of H is rarely reported
• May be important for diagnosis on small biopsy
  – effect of sampling in cystic/hemorrhagic/fibrotic areas
• Unknown impact in prognosis and resistance to drugs
  – Digital analyses + AI could be of help, to find useful « patterns »?
Intra-TH in NET different levels

• Morphology
• Function-phenotype
• Molecular alterations (including proliferation)
Molecular ITH in NET

• TH at the molecular level rarely reported in NET
  – Recent molecular results describe inter-patient H*
  – Research needed, using new tools « single cell analyses »

• In practice, Heterogeneity is important for current molecular indicators of prognosis
  – Proliferation+++
    • Ki-67: important marker, strong link with prognosis...and probably with other molecular markers

* Sadanandam et al. Can Discovery 2015; Scarpa et al. Nature 2017
ITH of proliferation

• Intra-T « spatial H »: Well known, reported in primary & metastases++
  – To be taken into account (counting in hot spots according to WHO for many years)
  – Areas of high Ki-67 of prognostic significance

• Inter-T « temporal H »: less reported
Intra-Primary (pancreas) H G1/G2

- **EUS-FNA** to diagnose pancreatic NET (N=46)
- Good interobserver agreement obtained for Ki67 (if >200 cells)
- EUS/FNA was able to distinguish a poor prognostic group with lower PFS
- Discrepancies in G2 tumours: FNA underestimate grading (G1 vs G2)

Ki-67 heterogeneity in primary may impact the diagnosis on FNA (due to sampling on G1 instead of G2 areas)

Intra-Metastases H G1/G2

Ki-67 Heterogeneity in liver met may impact the diagnosis on biopsy (grade may be underestimated, G1 vs G2)

Liver Metastasis

Virtual biopsies 1mm²

Biopsy: grade G2 area

Biopsy: grade G1 area

In whole slide=G2 (highest count)

Effect of Tumor Heterogeneity on the Assessment of Ki67 Labeling Index in Well-differentiated Neuroendocrine Tumors Metastatic to the Liver: Implications for Prognostic Stratification

Zhaohai Yang, MD, PhD, Laura H. Tang, MD, PhD, and David S. Klimstra, MD
- Also demonstrate that areas of high grade are of prognostic value

- When Ki-67 Heterogeneity is taken into account (by counting areas of «hot spot») better prediction of survival

- Important to identify the highly proliferating areas

Effect of Tumor Heterogeneity on the Assessment of Ki67 Labeling Index in Well-differentiated Neuroendocrine Tumors Metastatic to the Liver: Implications for Prognostic Stratification

Zhaohai Yang, MD, PhD, Laura H. Tang, MD, PhD, and David S. Klimstra, MD

Comparable results in ileal NET

Ki-67 proliferative index predicts progression-free survival of patients with well-differentiated ileal neuroendocrine tumors
Primary vs liver met, H G1/G2

Ki67 higher in synchronous liver metastases

Elevated Ki-67 labeling index in ‘synchronous liver metastases’ of well differentiated enteropancreatic neuroendocrine tumor

Pathology International 2013; 63: 532–538

Yoh Zen¹ and Nigel Heaton²
2 cases of Panc NET evolution with progression into G3 on successive resections

CASE 1
Primary tumor + LNodes
8% surgery
Liver Metastases 52%
Surgery

CASE 2
Primary tumor
15% surgery
Liver Metastases 16%
Mediastinal lymph node 15%
Peri-pancreatic lymph node 17%

Mediastinal mass 16%
Mediastinal lymph node 65%
Surgery
Surgery

First « indolent » controllable course then a more aggressive progression
• Demonstrate metachronous Heterogeneity of Ki-67
• NETG2 can progress to NETG3
• The greater the interval, the greater the increase
Molecular connection of Ki-67 heterogeneity?

Proliferation seen as result of sequential molecular transformations of NET, highlighting a process of clonal evolution with specific molecular background

• What did we learn from Ki-67?
  • Areas and subclones of high proliferation show that NET are biologically heterogeneous
  • These subclones are of prognostic significance

• What can we expect from Ki-67?
  • Probably connected with molecular alterations, driver of aggressivity that could be better understood to predict NET prognosis
CASE 2: Panc NET evolution with progression into G3

Primary tumor: WD NET
Liver Metasases: WD NET
Mediastinal lymph node: WD NET
Peri-pancreatic lymph node: WD NET


Mediastinal mass: WD NET
Mediastinal lymph node: WD NET

15% 16% 15% 17%

16% 65%

2014

Images: Dr Jérôme CROS
CASE 2: Panc NET natural clonal evolution with molecular alterations

<table>
<thead>
<tr>
<th>Year</th>
<th>Primary tumor</th>
<th>Liver Metasases</th>
<th>Mediastinal lymph node</th>
<th>Peri-pancreatic lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>WD NET</td>
<td>WD NET</td>
<td>WD NET</td>
<td>WD NET</td>
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<tr>
<td>2003</td>
<td>Surgery</td>
<td>Surgery</td>
<td>Surgery</td>
<td>Surgery</td>
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<tr>
<td>2004</td>
<td>Surgery</td>
<td>Surgery</td>
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<tr>
<td>2006</td>
<td>Surgery</td>
<td>Surgery</td>
<td>Surgery</td>
<td>Surgery</td>
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</table>

<table>
<thead>
<tr>
<th>Year</th>
<th>Mediastinal mass</th>
<th>Mediastinal Lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014</td>
<td>16%</td>
<td>65%</td>
</tr>
<tr>
<td>2017</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ploidy:2

Images: Dr Jérôme CROS
Molecular analyses of lung carcinoids G3

- Analysis of synchronous and metachronous genetic H of lung high grade carcinoids in 3 patients
- Molecular analyses (NGS+CGH on FFPE samples) of successive or intermingled samples allowed to describe alteration driving the transformation into high grade carcinoids.

Genomic landscape of pulmonary carcinoids with high grade progression. Jerôme Cros, et al. POSTER PS-22/006, ECP, Nice 2019
Carcinoid, but with more pronounced cellular atypia in areas of high proliferation

SURGICAL SAMPLE OF LOW GRADE TUMOUR Ki-67 1%

Normal Rb expression

SURGICAL SAMPLE OF HIGH GRADE METASTASIS Ki-67 52%

Loss of Rb expression

Comparison of paired samples:

In high grade:
CGH profiles with increasing genomic alterations:
• homozygous del of CDKN1B
• a deep del of RB1 (arrow), confirmed by loss of Rb expression

A TP53 mutation
Both samples have a carcinoid morphology

Ki-67 >20%

Comparison of paired samples:

**In recurrence:**
CGH profiles with increasing genomic alterations:
- an heterogeneous del of chr17 with loss of TP53.

A new fusion transcript of EIF3E (exon1) - RSPO2 (exon2) activating the WNT/βcatenin pathway
• We confirm, with this multiple sample analysis, **molecular heterogeneity** of high grade carcinoids, through space or time

• They develop from low grade carcinoids by accumulating NEC-like molecular alterations particularly involving *RB1* and *TP53*

POSTER PS-22/006, ECP, Nice 2019
Genomic landscape of pulmonary carcinoids with high grade progression. Jerôme Cros, et al.
They found a high genetic heterogeneity between Prim. and hepatic Met.

**Analyzed 5 ileal NET with WES:**
- Heterogeneity in mutations between Prim. and Met.
- Different amount of common/private mutations between cases
- Higher Allele Frequency in Met, suggesting a more polyclonal population in primary T

**Number of mutations**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Primary</th>
<th>Metastasis</th>
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<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>6 24</td>
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<td>2</td>
</tr>
<tr>
<td>1</td>
<td>7 13</td>
<td>7</td>
</tr>
</tbody>
</table>
Copy number of metastasis and primary were similar in P3 and P4, differed partially in P2 and P5, and were completely different in P1.
Highlight the importance of sample selection in Heterogeneous tumours:

Transcriptomic analysis performed to study inter-Patient H in Panc NEN
1 sample/patient (small, frozen, selected at macroscopy)
Clustering separated the 4 groups....but 4 NETG3 were grouped with G1/2

A: NET Ki67 ≤5%
B: NET Ki67 5-15%
C: NET Ki67 >15%
D: NEC

J Cros et al, Genomic and transcriptomic characterization of aggressive well differentiated pancreatic NET, ENETS 2018
Intra-T Heterogeneity of our samples was confirmed. CyclinD/E2F/RB pathway modified in aggressive NET with zonal heterogeneity.

Intra-TH can impact the results of research if the sample selection, at gross examination, is not accurate and not representative of the whole tumour.

J Cros et al, Genomic and transcriptomic characterization of aggressive well differentiated pancreatic NET, ENETS 2018.
Summary
ITH and NET

• Low morphological, functional, non clonal ITH
  – Low proportion of stroma, few immune cells (≠ exocrine); role of vessels?

• Molecular ITH is still poorly documented
  – High intra- and inter-TH of proliferation
  – High Ki-67 subclones are connected with molecular drivers of aggressiveness…research challenge to analyze pathology-driven spatial and sequential H

• Representativity of tissue samples used for diagnostic purpose is questionable
  – ITH complicates the accurate assessment of markers
  – Today there is still no alternative to the tissue approach
Conclusion

ITH is a major challenge for research/diagnosis in oncology

😢 an important source of irreproducibility

😊 may offer new targeted therapeutic opportunities

Proper tumour sampling & tools may help to characterize ITH, such as

- single cells analyses
  - New rapid sequencing technologies
- techniques in FFPE to analyze in situ molecular results with regard to morphology in ≠ areas
  - MALDI-imaging
  - NGS (detection of 1-5% of mutated alleles)
  - Others: array CGH, DNA meth, RNA-seq…
Future directions

• **AI and digital pathology** with quantitative analysis of ITH
  
• **Imaging biomarkers** to guide biopsy and overcome ITH
  • A coupled «radio-pathology » analysis

• **Liquid biopsy** is a promising approach to inform ITH in a and less invasive way
Thank you!